DEVELOPMENT OF GENETIC TRANSFORMATION SYSTEMS FOR PAPAYA

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ABSTRACT

We have developed transformation systems for papaya (*Carica papaya* L.) with the intention of genetically engineering virus resistance into the crop. Papayas in Hawaii and throughout the world are susceptible to papaya ringspot virus (PRV), and resistance is nearly nonexistent in advanced germplasm. Transformation of plants with viral coat protein genes has been shown to confer virus resistance in other crops. The coat protein gene of PRV, along with a kanamycin selection gene (*kan*) and a ß-glucuronidase reporter gene (*gus*A), were targeted into papaya. Papaya transformation has been achieved following the development of two methods for high frequency somatic embryogenesis and the subsequent delivery of genes into somatic embryos via the particle gun and *Agrobacterium tumefaciens*. Embryogenesis occurred after culturing meristematic tissues on Murashige and Skoog media containing 2.3 to 113.1 μM 2,4-dichlorophenoxyacetic acid. The meristematic tissues consisted of immature zygotic embryos explanted about 100 days post-anthesis and hypocotyl sections from seedlings about ten days old. Embryogenic cultures regenerated normal-looking plants on media devoid of growth regulators. Evidence is presented for the insertion and/or expression of a chimeric gene for the coat protein of papaya ringspot virus (cpPRV), for kanamycin resistance, and for ß-

glucuronidase (GUS). There was 100% correspondence between selective growth of embryos, enzyme assays, and polymerase chain reaction (PCR) amplification of *kan* sequences. Expression of GUS was detected in about a third of the particle-bombarded samples, in contrast, all of the *A. tumefaciens*-transformed isolates expressed the reporter gene. PCR amplification of cpPRV sequences and Southern hybridization of genomic DNA to a cpPRV probe were observed in nearly all GUS-positive samples tested to date. A PCR-amplified sequence for *kan* from plants transformed with *A. tumefaciens*, was larger than the sequence amplified from the plasmid control and from plants transformed with the particle gun. The highest yield of transgenic isolates was 1.38% of the bombarded zygotic embryos. About 70% (23) of the isolates from zygotic embryos regenerated normal-looking plants while only 20% of the isolates from embryogenic calli produced plants.

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PREFACE

The research chapters in this dissertation are written in the format required by the journal *Plant Cell Reports* published by Springer-Verlag, Heidelberg, Germany. Two of the chapters have been published. Chapter 3, "Somatic embryogenesis and plant regeneration from immature zygotic embryos of papaya (*Carica papaya* L.)" appeared in *Plant Cell Reports* (1990) 9:320-324 and was co-authored by Maureen M. M. Fitch and Richard M. Manshardt. Chapter 5, "Stable transformation of papaya via microprojectile bombardment" appeared in *Plant Cell Reports* (1990) 9:189-194 and was co-authored by Maureen M. M. Fitch, Richard M. Manshardt, Dennis Gonsalves, Jerry L. Slightom, and John C. Sanford.

The wordprocessing program Microsoft Word 5.5 was used for the text and graphs were generated with Sigmaplot 4.0. The text and graphs were printed with a Hewlett-Packard Laserjet III printer. The photographs were taken with a Zeiss Stereoscope dissecting microscope fitted with a M63 35 mm camera, a Nikon FE 35 mm camera with a 55 mm macrolens, or a Polaroid MP-4 camera system. Color reproductions were made from slides taken with Kodak Ektachrome 160 film; black and white photographs were taken with Kodak TMAX 100 film or Polaroid Type 55 film.

CHAPTER 1. INTRODUCTION

Papaya is a popular breakfast and dessert fruit in many countries and is a daily vegetable staple in Southeast Asian communities. The crop is widely grown in orchards or as dooryard trees in many tropical and subtropical countries.

Production of papaya is severely limited in most areas, because the crop is highly susceptible to papaya ringspot virus (PRV), and virtually no resistance has been found in commercial cultivars. Developing virus resistant cultivars through conventional breeding and selection is a long-term endeavor, while current disease control practices like geographical isolation, sanitation, and cross protection provide only short-term solutions that salvage some profit for the grower.

Hawaii is the largest producer of papayas in the United States. Expanding urban areas in Hawaii, which act as reservoirs of PRV inoculum, are an increasing threat to the protection afforded by isolation and sanitation. Cross protection, a deliberate infection of plants with a mild strain of virus, is also used in Hawaii to prevent infection by virulent strains, although the mechanism is not understood at this time. Cross protection provides economic benefits only in areas with a high incidence of PRV. Furthermore, breakdown in protection can occur if the virus

is introduced to vulnerable areas of the plant, for example, the young shoots.

Gene transfer techniques for plant virus resistance exist and could enhance breeding programs by ameliorating disease problems more quickly. Transformation of plants with viral coat protein genes has resulted in genetically engineered virus resistance in tobacco, tomatoes, and potatoes (Beachy et al. 1990, Grumet 1990, Nejidat et al. 1990). Torrey (1985) remarked that "one of the most attractive prospects for the future is the use of cultured plant tissues, cells, or protoplasts as receptors for selected cloned DNA conveying specific genetic information which can thereafter be expressed either in the cultured tissue or cells or in organized plants derived from the cultured cells. This approach combines the methods of plant tissue culture and the capabilities of genetic engineering." Torrey's comment accurately describes the objectives in this study, which are 1) to develop an efficient regeneration system for papaya tissue culture, 2) to transform the regenerable papaya cultures and produce transgenic plants, and 3) to confirm and characterize the transgenic plants. The genes that were transferred into papaya were the coat protein gene of papaya ringspot virus, a selection gene, and a reporter gene. The coat protein gene may protect papaya from PRV, and the latter two genes are valuable for identifying transgenic plants.

While resistance to PRV provides the rationale for developing a genetic transformation system for papaya in this dissertation, the technique would also be valuable for the transfer of any gene that might result in crop improvement, for example, fungus or fruit fly resistance genes.

CHAPTER 2. LITERATURE REVIEW

2.1 PAPAYA

Papaya (*Carica papaya* L.) is a perennial tropical and subtropical tree crop that is favored as a breakfast or dessert fruit in Western cultures and as a vegetable staple among certain Southeast Asian populations, for example, in Thailand. Worldwide annual production is 3.87 million metric tons (FAO 1990). About half of the production is centered in South America (1.8 million metric tons) and about one-fourth of the crop is produced in Asia (1 million metric tons). Developing market economies account for 95% of the production. Fresh fruit as well as papain, a proteolytic enzyme, are the major products, although pulp for juice additives has some economic importance (Litz 1986b).

Papaya belongs to the Caricaceae, a family of dicotyledonous tree-like plants that is made up of four genera, three of which, *Carica, Jacaratia,* and *Jarilla*, are native to tropical America (Badillo 1967, 1971). *Cylicomorpha* is native to Africa (Purseglove 1968). Members of the Passifloraceae are among the nearest relatives (Lawrence 1951). The genus *Carica* consists of about 21 species but only *C. papaya* is of economic importance (Storey 1953). The ripe fruit of other species are edible, for

example, *C. pubescens* Lenne et Koch, *C. monoica* Desf., *C.* x *heilbornii* Badillo nm. *pentagona* (babaco), *C. quercifolia* St. Hil (Heiron.), and *C. goudotiana* Solms-Lauback (Litz 1986b), but they are more often consumed in preserves (Storey 1976).

Storey (1953) reviewed much of the basic information about papaya. The chromosome number of all *Caricas* is 2x = 2n = 18. Papayas are classified by flower morphology into three sex types, staminate, pistillate, and hermaphrodite, and flowers are arranged in cymes that are borne in the axils of palmately lobed, hollow-stemmed leaves. Papaya flowers in staminate and hermaphrodite trees are sensitive to seasonal temperature fluctuations, becoming carpellodic at low temperatures and abortive at high temperatures. Round fruit are produced by pistillate plants, while hermaphrodite plants are characterized by ovoid or pyriform fruit. Papaya flowers are produced as early as four months after germination from seed, and fruit ripen in nine to 14 months. The flesh of ripe fruit is yellow to red. Selections are made for a pleasing melon-like fragrance and taste. Ripe papayas are rich in vitamin A and C (Litz 1986b). The enzyme papain is produced in laticifers that are functional throughout the plant in all tissues except for the ripe fruit (Litz 1986b).

Plants grow to a height of 3 to 8 m (Foster 1943). The semi-woody, hollow-stemmed trunk usually supports a single

apex apparently due to strong apical dominance, however, old specimens can attain the age of 25 years and can become broad masses of multilateral branches (Storey 1953). Plantations normally retain trees for three to four years, during which time production is continuous. After that period, the trees are too tall to harvest or they succumb to diseases that decrease production. In Hawaii, about 74 million pounds of fruit were harvested from 2500 acres in 1989, from four of the islands (State of Hawaii Department of Agriculture 1990). The bulk of the crop was grown on the island of Hawaii (2374 acres). Papaya, valued at about $14.4 million in 1989, was the fifth most important crop after sugarcane, pineapples, macadamia nuts, and potted foliage plants. Papayas represented about 3% of the total crop value in the state. About two-thirds of the crop was exported to the U. S. mainland and to Japan, while one-third was consumed locally.

Papayas in Hawaii are beset by several pest problems. The Mediterranean fruit fly (*Ceratitis capitata*), melon fly (*Dacus cucurbitae*), and Oriental fruit fly (*Dacus dorsalis*) oviposit eggs into ripening papaya fruit; thus, post-harvest treatment of fruit is necessary (Armstrong et al. 1989). Although fumigation with ethylene dibromide (EDB) had been effective, it was banned from use in 1984 (Anonymous 1984). Regulations governing importation of papayas in the US Mainland and Japan require post-harvest treatment of the

fruit. The current "double-dip" hot water treatment (Couey and Hayes 1986) will be supplanted by the newer high-temperature, forced-air heat treatments (Armstrong et al. 1989) for shipments to the US Mainland.

2.1.1 THE VIRUS PROBLEM AND CURRENT SOLUTIONS

Papaya is beset by two major pathogens in Hawaii, the fungus, *Phytophthora palmivora* Butl. (Alvarez and Nelson 1982), and papaya ringspot virus (PRV, Purcifull 1972). Worldwide, PRV is also of major importance (Purcifull 1972). Resistance to PRV in the commercial genotypes is nearly nonexistent (Conover and Litz 1978), and breeding for resistance using exotic germplasm has been a long-term project involving wide hybridization with exotic species (Manshardt and Wenslaff 1989a, 1989b).

Virus problems in Hawaiian papayas have been documented since 1938 (Parris), but the identity of viruses was difficult to establish without specific serological tests and electron microscopy. Gonsalves and Ishii (1980), using both methods, identified the virus in Hawaiian papaya as PRV, a potyvirus. PRV is the most severe virus disease of the crop, and another, papaya mosaic virus (PMV), is of minor importance (Purcifull and Hiebert 1971). Confusion in the literature exists because PRV is often called "papaya mosaic" due to the mottled symptoms of the disease on the

leaves. PMV is an unrelated virus of the potexvirus group (Purcifull and Hiebert 1971). PRV is serologically identical to watermelon mosaic virus type 1 (WMV-1), renamed PRV-w, that infects cucurbits but not papaya (Yeh and Gonsalves 1984b, Yeh et al. 1984). PRV particles are flexuous rods about 780 x 12 nm, containing positive sense, single-stranded, monopartite RNA genomes of MW about 330,000 (Purcifull et al. 1985). The symptoms caused by PRV make its diagnosis fairly simple. Ringspots that appear to be water-soaked develop on the green epidermis of immature fruit, and linear lesions occur on the younger portions of stems and petioles (Conover 1964a, Yeh et al. 1988). The leaves exhibit a mottled appearance due to intermingling areas of dark-green and light-green lamina tissues (Conover 1964a). Environmental temperature affects symptom development which varies with season (Gonsalves and Ishii 1980). The warm summertime temperatures of Hawaii are correlated with low virus titer. Some infected plants appear to be symptomless and exhibit negative enzyme-linked immunosorbent assay (ELISA) results. Viral replication is apparently stimulated by the cooler temperatures of the winter months when both virus titer and symptom severity are high. Virus infection causes gradual decline and eventual death of the tree; thus, roguing of trees exhibiting symptoms is a major form of control (Namba and Higa 1977).

2.1.2 BREEDING FOR RESISTANCE

The most effective method for solving a disease problem like PRV is to breed for resistance (Hawkes 1983, Grumet 1990). Limitations exist. For example, the resistance gene(s) may not exist in the crop, breeding may be difficult if a quantitative character is involved, and the character(s) may be linked to undesirable traits like slow growth, toxicity, or susceptibility to other factors (Grumet 1990).

A dioecious papaya cultivar 'Cariflora', selected in Florida (Conover et al. 1986), shows tolerance to PRV. Zee (1985) crossed a sib line of 'Cariflora' with Hawaiian cultivars to produce hermaphrodite progeny. The F1 and F2 populations were selected for PRV resistance and acceptable fruit quality. Resistance in such crosses had been found to be inherited quantitatively (Conover and Litz 1978). Levels of resistance equal to the Florida parent were expressed in F2 selections from a cross with 'Higgins'; however, fruit quality was poor (Zee 1985). 'Kapoho' and 'Sunrise' progeny showed higher quality, and selections from F2 and F3 progeny are planned.

If resistance traits do not exist in commercial cultivars, plant breeders turn to unimproved germplasm; for example, wild species often possess resistance to disease

(Hawkes 1983). Wide hybridization and recurrent backcrossing have resulted in limited introgression of disease resistance genes from wild tomatoes into commercially acceptable genotypes (Rick 1960, 1983). Horovitz and Jimenez (1967) crossed *Carica* species differing in PRV resistance to determine heritability and type of resistance. They observed that progeny from crosses of susceptible *C. monoica* Desf. with resistant *C. pubescens* Lenne et Koch and *C. cauliflora* Jacq., when backcrossed and test-crossed, segregated for PRV resistance in a manner that suggested that resistance is controlled by a single dominant gene. Provvidenti and Robinson (1977) and Provvidenti and Gonsalves (1982) observed similar results in crosses between wild cucurbits. Resistance has also been identified in the wild species *C. candicans* A. Gray (Riccelli 1963). Manshardt and Wenslaff (1989a, 1989b) crossed papaya with seven wild species, four of which have been reported to be virus resistant. They rescued embryos of interspecific hybrids from crosses with resistant *C. pubescens* (Larter 1938, Conover 1964a, Horovitz and Jimenez 1967), *C. quercifolia* (Conover 1964a, Horovitz and Jimenez 1967), *C. cauliflora* (Malaguti et al. 1957, Reynolds 1959, Conover 1964a), and *C. stipulata* Badillo (Horovitz and Jimenez 1967). The sterility barrier to wide hybridizations was attributed to failure of hybrid endosperm development. Hybridization with papaya failed with two species, *C.*

monoica and *C. parviflora* (A. DC.) Solms, and one natural hybrid, *C.* x *heilbornii* Badillo nm. *pentagona* (Heilborn) (Manshardt and Wenslaff 1989b). Others have produced progeny from interspecific crosses as well (Khuspe et al. 1980, Litz and Conover 1981b, 1982, 1983). Manshardt (unpublished data) showed that F1 populations exhibited field resistance to PRV symptom development; however, the hybrids were nearly infertile, having less than 1% stainable pollen. Fruit of the interspecific hybrids were small, and years of backcrossing are probably required before a commercially acceptable product could be available. Classical breeding methods may lead to broad and single gene resistance.

2.1.3 QUARANTINE, SANITATION, GEOGRAPHIC ISOLATION

Other methods to combat the virus disease rely on current quarantine and sanitation practices that allow economic survival of the crop in Hawaii (Namba and Higa 1977) and elsewhere. The virus is vectored by winged individuals of the green peach aphid, *Myzus persicae* (Jensen 1949a, 1949b), that randomly probe and infect papaya trees with virus-contaminated stylets in a nonpersistent manner (Namba and Kawanishi 1966). Control of aphids is not practicable (Adsuar 1946, Conover 1964a, Namba and Kawanishi 1966). Virus-free zones exist in geographically isolated

areas (Namba and Higa 1977); however, urban growth threatens the isolation. The island of Oahu accounts for 76% of the 1.1 million population of the State of Hawaii (State of Hawaii, Department of Business, Economic Development, and Tourism 1990) and supports only small, isolated papaya orchards. The potential virus inoculum from infected trees in homeowners' yards is high. The virus-free Puna area on the island of Hawaii faces imminent threat as the population center expands from the urban Hilo area that harbors the virus and the vector. Sanitation, quarantine, and roguing of infected individuals are the major protective measures in these areas (Namba and Higa 1977).

2.1.4 CROSS PROTECTION

Cross protection is a plant protection strategy that can be implemented in areas like Oahu where virus inoculum density is high. In cross protection, a crop is deliberately infected by a mild strain of virus, and systemic infection enables plants to survive challenge by virulent strains in the field (Fulton 1986). Cross-protected plants exhibit mild virus symptoms; the mechanism of protection has not been elucidated although several hypotheses have been presented (Sherwood and Fulton 1982, Palukaitis and Zaitlin 1984). Sherwood and Fulton (1982) suggested that the coat protein of tobacco mosaic virus

(TMV) may be directly responsible for cross protection against TMV. Palukaitis and Zaitlin (1984) postulated that the positive, sense-strands of viral RNA from the protecting strain bind to the genomic template antisense strand of the incoming virus, and that mechanism inhibits further viral replication.

Cross protection is used in commercial applications on high-value horticultural crops like greenhouse-grown tomatoes (Fletcher 1978), citrus (Costa and Muller 1980), and papaya (Yeh and Gonsalves 1984a, Yeh et al. 1988) that are infected by tomato mosaic virus, citrus tristeza virus, and PRV, respectively. Cross protection is implemented in areas where, due to high infection pressure, neither roguing nor sanitation are effective. In the case of PRV, the mild strain of virus, HA 5-1, was isolated after nitrous oxide treatment of the virulent Hawaiian PRV strain HA 5 (Yeh and Gonsalves 1984a), and it has been used in commercial applications in Taiwan (Wang et al. 1987, Yeh et al. 1988). Yeh and Gonsalves (1984a) determined that the time interval between protection and challenge as well as the position on the plant that is challenge inoculated, determine the efficiency of cross protection. The time interval had to be at least 26 days for optimum protection, and challenge inoculation in the uppermost unexpanded leaf resulted in 80% superinfection. The strain of mild virus was also important (Yeh and Gonsalves 1984a). The experiments showed that

systemic protection must be established before cross protection is effective. Cross protection has been implemented on large scale and resulted in increased income in Taiwan (Yeh et al. 1988) and Hawaii (D. Gonsalves, unpublished data). Superinfection eventually occurs in experimental and commercial plots. The youngest, fast-growing regions of the tree apex are apparently not protected (Yeh and Gonsalves 1984a). These tissues are known to free of virus, a phenomenon used to advantage in isolating virus-free tissue cultures (Quak 1977). Apparently, viruses cannot replicate rapidly enough to remain in high titer in the apical region, and/or there is some component of the apical region that precludes the presence of virus. Superinfection can negate cross protection if symptom development becomes severe. The hypothesis is that once the invading virus uncoats, the virus can become established despite the presence of the cross protecting virus. Besides breakdown in protection, other disadvantages of cross protection are that a mild strain of virus must be located or produced by mutagenesis as described for PRV and that some yield loss due to the presence of the mild strain may occur in the crop. Other crops in the vicinity may become severely affected if they are hosts to the virus. The virus may cause severe symptoms via synergism with other viruses or it may mutate in the field to a severe form. This would not be a problem if the

field were in an area where the inoculum from severe strains were already high. Yeh et al. (1988) commented that the mild strains, HA 5-1 and HA 6-1, appeared to be stable in the field because no revertants were observed. Additional labor is involved in inoculating field material prior to planting (Fulton 1986).

2.2 GENETICALLY ENGINEERED VIRUS RESISTANCE

Genetically engineered protection of crops using pathogen-derived genes was suggested by Sanford and Johnston (1985). They hypothesized that viral coat protein (CP) and/or replicase could compete for plant cellular components and thus inhibit establishment of the challenge virus. The hypothesis was based on results with the bacteriophage QB, where the genes involved in pathogen replication (replicase complex genes) and the CP gene protected bacteria from virus infection. They reasoned that pathogen-derived resistance would be more stable because pathogens would be less likely to mutate against genes derived from their own genomes. Their hypothesis was supported with the work of Powell Abel et al. (1986) who transformed tobacco with the engineered CP gene of tobacco mosaic virus (TMV) and transgenic plants showed delayed symptom development after deliberate challenge inoculation. Plant virus resistance via genetic engineering became a fertile area of research, and the

mechanism of CP protection had been postulated to be analogous to cross protection (Powell Abel et al. 1986). However, as more data is accumulated from various groups transforming plants with viral CP genes, the evidence suggests that the analogy is not so simple. For example, Beachy's group (Stark and Beachy 1989) has observed that potyvirus CP affords a wide range of protection against several related potyviruses whereas in classical cross protection, the same does not hold true. Of significance are findings that CP genes from different types of viruses show some degree of protection to plants regardless of the genome structure of the virus (Anderson et al. 1989).

2.2.1 ANTISENSE RNAS

Coat protein-mediated resistance is, by far, the most widely used method; however, others of varying effectiveness have been described. Viral CP genes in the antisense orientation have been engineered into plants, but the degree of protection with the antisense RNA was low compared to CP protection (Cuozzo et al. 1988, Hemenway et al. 1988, Powell et al. 1989). Antisense technology has been used in animal, microbial, and plant systems to inhibit gene expression by unknown mechanisms (Grumet 1990). Antisense sequences may hybridize with complementary mRNA strands, limiting their access to ribosomes for translation, or RNA:RNA hybrids may

be selectively degraded (Nejidat et al. 1990). The antisense RNA may interfere with replication since the plus-strand serves as a template for minus-strand synthesis (Cuozzo et al. 1988). Powell et al. (1989) postulated that antisense protection is due to the disruption of the 3′ binding site of the putative viral replicase. Rezaian et al. (1988) made antisense constructs from each of the three cucumber mosaic virus (CMV) RNAs and found that the 5′ ends were important for initiation of translation while the 3′ ends were important for initiation of replication. However, except for one plant, transformants were not resistant to CMV. Cuozzo et al. (1988), Hemenway et al. (1988), and Powell et al. (1989) reported limited protection with antisense CP genes for CMV, potato virus X (PVX), and TMV, respectively. They compared antisense with sense constructs of CP genes and reported similar results. In the work with CMV, there was 70 to 100% protection with CP gene transformants challenged with high virus inoculum (25 μg ml^{-1} virus), while there was only 50% protection in antisense transformants challenged with low inoculum (1.0 μg ml^{-1} virus). Protection against PVX was 70 to 90% with CP gene transformants subjected to high virus inoculum (5.0 μg ml^{-1}), and the same level of protection in antisense transformants was demonstrated with only a low inoculum (0.05 μg ml^{-1}). Powell et al. (1989) showed that the CP gene protected transformed plants 100% against both low and

high inoculum concentrations of TMV (0.01 and 0.05 $\mu g\ ml^{-1}$), but antisense protection was 80 to 95% and 5 to 40% with low and high inocula, respectively. Thus, CP antisense gene transformation protects plants from three viruses at low inoculum challenge.

2.2.2 SATELLITE RNAS

Several viruses have parasites called satellite RNAs that are about 200 to 300 nucleotides in size and that do not share sequence homology with the viruses themselves (Grumet 1990). Satellite RNAs use the viral replication genes of the virus they parasitize and coat themselves with viral coat protein (Harrison et al. 1987, Gerlach et al. 1987). Only a few viruses have the parasites, and the effects of the satellites are unpredictable, sometimes they attenuate symptoms, sometimes they increase them (Harrison et al. 1987). Satellite sequences of tobacco ringspot virus (ToRV, Gerlach et al. 1987) or CMV (Harrison et al. 1987) were engineered into tobacco for effective protection if there was more than one gene copy per genome. Superinfection did not occur in either experiment. It is doubtful that satellite RNAs encode polypeptides (Nejidat et al. 1990). In CMV, satellite transformation reduced virus titer 80 to 95% in tobacco; however, satellite RNA made by the tobacco was picked up by an invading, unsatellited

virus. The risk due to unknown consequences of such a response is a disadvantage of satellite protection. Disarming satellites to curtail their unpredictability has been discussed (Grumet, 1990).

2.2.3 VIRUS GENOME

The entire viral genome of a mild strain of TMV was engineered into tobacco and was shown to be effective as genomic cross protection (Yamaya et al. 1988). Disease symptoms were reduced or eliminated with this heritable cross protection. In contrast to CP-protected plants, superinfection and protection breakdown did not occur after challenge with uncoated viral RNA. The level of protection was higher than with CP alone, a challenge inoculation of 5.0 μg ml^{-1} did not cause superinfection, whereas plants transgenic for CP alone could only withstand challenge inoculations of 0.2 to 0.5 μg ml^{-1}. The engineered virus replicates autonomously in the protected host, possibly resulting in higher concentrations of the protective agent compared to constitutively produced CP (Grumet 1990). However, some risk is entailed because a whole virus is synthesized by the plant with unpredictable consequences. Producing a disarmed deletion mutant has been discussed. Engineered cross protection shares the same disadvantages as classical cross protection, a mild strain of virus must be

available to engineer into a plant, the mild strain may mutate, and yield losses may be consequences of protection.

2.2.4 DEFECTIVE INTERFERING RNAS

Defective interfering RNAs are similar to satellite RNAs because they depend on the invading virus for replication, use its CP, and can cause attenuated disease symptoms (Grumet 1990). Defective interfering RNAs don't share sequence homology with the helper virus but somehow they interfere with normal functioning of the invading (helper) viral RNA; thus, they serve a protective function to the plant (Grumet 1990). The native virus RNA replicase system is apparently disrupted.

2.2.5 VIRAL REPLICASE GENES

A new and apparently highly effective protection mechanism is to engineer the replicase complex of a virus into a plant. Golemboski et al. (1990) cloned part of the putative replicase complex of TMV and transformed it into tobacco plants. The complete resistance that they observed was specific to the virus strain and degree of resistance was ten times higher than that afforded by CP-mediated protection. Transgenic plants were resistant to 300 $\mu g\ ml^{-1}$ of infective virus particles or 500 $\mu g\ ml^{-1}$ viral RNA. The

54 kD protein product of the gene could not be isolated. It is believed to be either quickly assimilated into some function or it is extremely labile. This nonstructural gene did not protect plants from distantly related virus strains. The mechanism of protection is not understood; however, it has been postulated that the plant-produced replicase or the DNA that codes for it compete for components in the cell. Apparently this interferes with the ability of the invading virus to replicate.

2.2.6 ANTIBODY GENES

Finally, an intriguing idea is to arm plants with antibody-producing genes that would enable them to bind invasive antigens and immunize plants in a manner analogous to mammalian systems. Hiatt et al. (1989) transformed tobacco lines with immunoglobulin cDNA sequences from mouse hybridoma mRNA and obtained transformants that expressed either the light gamma or heavy kappa immunoglobulin chains. The two types of transformants were crossed, yielding some hybrids that expressed both chains and made functional antibodies. Antigen binding by transgenic plant antibodies was equivalent to comparable native immunoglobulins. Düring et al. (1990) transformed tobacco with a construct for production of both light and heavy chains in transgenic

plants. Assembled antibodies were detected in both endoplasmic reticulum and chloroplasts of tobacco.

2.2.7 COAT PROTEIN-MEDIATED VIRUS RESISTANCE

Coat protein-mediated virus resistance has been shown to be effective for at least eight different viruses (Beachy et al. 1990, Kawchuk et al. 1990). The method developed by Powell Abel et al. (1986) was found to be generally applicable (van den Elzen et al. 1989, Grumet 1990, Nejidat et al. 1990, Beachy et al. 1990). Their technique involved the isolation, cloning, and sequencing of the CP gene from the sense strand of the viral RNA, followed by the construction of a chimeric gene that could be expressed in plants. The construct consisted of the viral CP coding sequence inserted between the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase *(nos)* 3′ termination sequences. Tobacco was transformed with *Agrobacterium tumefaciens* using the leaf disk method (Horsch et al. 1985). Since 1986, others have corroborated the general utility of CP-mediated protection; however, differences in the extent of protection have been documented (Grumet 1990, Nejidat et al. 1990).

Virus resistance has been defined as delay in or escape from disease symptoms compared to controls, decrease in severity of symptoms and virus titer, or reduction in local

lesion numbers on local lesion host (Nejidat et al. 1990). Expression of CP did not have a negative effect on growth, fertility, or morphology of transgenic plants (Grumet 1990).

Powell Abel et al. showed that virus symptom delay was correlated with TMV inoculation concentration (1986, 1990) as well as copy number of the gene. Correlation of degree of protection with copy number was verified by Loesch-Fries et al. (1987) who found greater protection in plants harboring more than one copy of the transgene. Others showed no correlation between copy number and degree of protection in potato virus Y (PVY) (Lawson et al. 1990). Higher CP expression in potatoes rather than gene copy number was found to give greater protection against potato virus X (PVX) in plants containing equal numbers of gene copies (Hemenway et al. 1988, Hoekema et al. 1989). In another study, the relative concentration of CP (w/w) was equivalent in two transgenic tobacco plant lines, but there was a difference in the level of protection attributed to the fact that the transgenes for CP were driven by different promoters. Clark et al. (1990) showed that plants containing CP genes constructed with the CaMV 35S promoter were more highly resistant than ones transformed with CP driven by a ribulose bisphosphate carboxylase small subunit (*rbc*S) gene promoter. Tissue specificity that is directed by the *rbcS* gene promoter may influence level of protection by differential expression of CP. Defective CP transcripts

were not protective for AlMV (van Dun et al. 1988) nor TMV (Powell et al. 1990), suggesting that the gene product rather than the transcript was the protective factor.

The collective data from several groups show that tobacco can be protected with CP genes from eight different types of viruses. Resistance was conferred with CP from soybean mosaic virus (SMV, potyvirus, Stark and Beachy 1989), tobacco streak virus (TSV, ilarvirus, van Dun and Bol 1988), tobacco rattle virus (TRV, tobravirus, van Dun and Bol 1988), PVX (potexvirus, Hemenway et al. 1988), CMV (cucumovirus, Cuozzo et al. 1988), AlMV (alfalfa mosaic virus, Tumer et al. 1987, Loesch-Fries et al. 1987, van Dun et al. 1987), potato leafroll virus (PLRV, luteovirus, Kawchuk et al. 1990) and TMV (tobamovirus, Powell Abel et al. 1986). All groups showed delay in systemic disease development in CP-positive plants compared to CP-negative plants (Nejidat et al. 1990, Beachy et al. 1990, Grumet 1990, Kawchuk et al. 1990).

In addition, CP-mediated protection exhibited some breadth in effectiveness against related and unrelated viruses. Nelson et al. (1987) found that the CP of TMV U1 strain protected tobacco from PV230, another severe TMV strain, and three more strains of tobacco mosaic virus. The CP of AlMV protected tobacco from another AlMV strain (Loesch-Fries et al. 1987) and the CP of tobacco rattle virus protected tobacco from pea early browning virus, a

related virus (PEBV, van Dun and Bol 1988). Tobacco plants transgenic for AlMV CP showed delay in disease development of PVX and CMV but were not protected against TMV U1 strain (Anderson et al. 1989). TMV CP protected tobacco plants from PVX, PVY, and CMV, although to a lesser extent (Anderson et al. 1989). Protection was conferred against AlMV but not TMV with the AlMV CP gene (van Dun et al. 1987, Loesch-Fries et al. 1987). Van Dun and Bol (1988) showed that tobacco rattle virus strain TCM protected against the highly homologous pea early browning virus but not against the heterologous (39% homology) TMV strain PLB.

Potyvirus CP apparently confers broad protection. Stark and Beachy (1989) showed that soybean mosaic virus (SMV) CP protected tobacco from two other potyviruses, tobacco etch virus (TEV, 61% homology) and potato virus Y (PVY, 58% homology). In a similar study using TMV CP, Nejidat and Beachy (1990) determined that sequence homology between protective CP and invading virus were correlated with degree of resistance. High resistance was shown to tomato mosaic virus (ToMV), pepper mild mottle virus (PMMV), tobacco mild green mosaic virus (TMGMV), and odontoglossom ringspot virus (ORSV), tobamoviruses that share 82 to 60% homology with TMV CP. Ribgrass mosaic virus (RMV, 45% homology) was afforded only weak protection. Apparently, 60% homology is the lower limit for conferring strong protection. However, it is not known if homology in

specific structural domains is important (Nejidat et al. 1990).

Coat protein accumulation in transgenic plants is variable, levels from 0.001 to 0.2% of total soluble leaf protein have been reported (Grumet 1990, Beachy et al. 1990, Nejidat et al. 1990). The variation in expression level has been attributed to gene copy number, position of gene insertion, or somaclonal variation (Larkin and Scowcroft 1981) in the regenerated plants (Nejidat et al. 1990).

The degree of protection, independent of CP expression level, has been reported to be variable, too, and is apparently dependent on plant species and temperature (Nelson et al. 1988, Nejidat and Beachy 1989). Environmental effects are known to cause large differences in plant response to virus infection (Matthews 1981). Nelson et al. (1988) showed that transgenic tomatoes were virus resistant under field conditions; however, CP concentration decreased. Nejidat and Beachy (1989) showed that resistance to TMV in tobacco but not tomato was affected by temperature. Under high temperatures, 30-35^{o}C, CP concentration decreased 90% in both tobacco and tomato, but virus resistance decreased only in tobacco. For these reasons, virus resistance testing is recommended for transgenic plants of different physiological ages both in the greenhouse and field before a resistance rating is given (Nejidat et al. 1990).

Viral RNA in classical cross protection can cause superinfection, and the phenomenon occurred in some cases of CP-mediated protection, too. TMV (fully or partially uncoated) and AlMV RNA overcame CP resistance (Powell Abel et al. 1986, Nelson et al. 1987, Nejidat and Beachy 1990, Register and Beachy 1988) but treatment with PVX RNA did not overcome PVX CP protection (Hemenway et al. 1988).

Little is known about viral uncoating. TMV is thought to be co-translationally disassembled in "striposomes" made of ribosomes (Shaw et al. 1986). The structure of "striposomes" in transgenic plants is not known. Since the heterologous protection by TMV CP is correlated with CP amino acid sequence homology, a receptor moiety may be part of the uncoating site. CP sequence determines a basic conformation of the CP which may competitively block access of the virus to the receptor site, or the CP may re-encapsidate the partially uncoated virus (Nejidat et al. 1990).

Nejidat et al. (1990) mentioned several areas for future investigations. Coat proteins from different types of viruses may protect differently. Mutation of the CP should provide information on the essential domains for CP-mediated protection. Finally, portions of essential domains could be joined and/or amplified for even greater CP-mediated protection.

Constructs for CP-mediated resistance were delivered into tobacco, tomato, and potato plants using *A. tumefaciens*. Transgenic plants were recovered by leaf disk or potato tuber organogenesis on selective media as described by Horsch et al. (1985). The *A. tumefaciens* strains consisted of the disarmed C58 nopaline- or A281 octopine-types hosting Ti plasmids that were cointegrates or helpers in binary constructs. The transgenic plants were selected for aminoglycoside resistance in tissue cultures with the antibiotic kanamycin. In several cases, the R0 plants (original transgenic isolates) were self-pollinated, and the progeny (R1) segregated for inserted sequences including the coat protein genes (Powell Abel et al. 1986, 1990, van Dun et al. 1987, Tumer et al. 1987, Loesch-Fries et al. 1988). Mendelian segregation was observed, suggesting that one or a few gene copies had been inserted into the plants' genomes.

The following section will review transformation techniques that helped in the design of experiments for transferring CP, selection, and reporter genes into papaya cells.

2.3 TRANSFORMATION

Plant transformation has been accomplished by many unique methodologies, but the most well-known one is

fascinating in its complexity, and it occurs in nature. *Agrobacterium tumefaciens* and *A. rhizogenes* are bacteria that are able to insert their extrachromosomal genes into plant genomes, causing crown gall and hairy root disease, respectively (Braun 1982, Chilton et al. 1977). *Agrobacterium tumefaciens*-mediated transformation along with microprojectile bombardment will be reviewed in detail, since transgenic papaya plants were produced using those methods. Several other protocols have yielded transgenic plants as well. *Agrobacterium*-mediated transformation has been the method of choice with dicot species; most of the other methods have been implemented for monocot species because monocots are not easily infected by *Agrobacterium*.

2.3.1 DIRECT DNA UPTAKE

One of the earliest means of tranferring foreign genes into plant cells was accomplished with direct uptake of DNA by protoplasts (Paszkowski et al. 1984). The barrier imposed by the tough cell wall was removed with cellulose-degrading enzymes without loss of the protoplasts' ability to re-grow cell walls and re-form calli (Cocking 1960). DNA in the form of *A. tumefaciens* Ti plasmids or other types of plasmids was mixed with protoplasts and polyethylene glycol (PEG) to facilitate DNA uptake and transgenic tobacco plants were regenerated that showed Mendelian segregation of the

kanamycin resistance gene (Paszkowski et al. 1984). An electric charge put across the cultures also caused tobacco cells to take up DNA through holes in the plasmalemma (electroporation), and transgenic plants were recovered as well (Fromm et al. 1985). Uptake of DNA by rice protoplasts has been shown to be highly efficient. Several laboratories have demonstrated rice transformation by electroporation (Toriyama et al. 1988, Zhang et al. 1988, Shimamoto et al. 1989) or PEG-mediated uptake (Zhang and Wu 1988) even in the recalcitrant *Oryza sativa* var. *indica* varieties (Datta et al. 1990). The importance of the cereals as major food crops has fueled research with the result that many plant species have been transformed via protoplast uptake of DNA (Potrykus et al. 1985a, 1985b, Paszkowski et al. 1984, Lörz et al. 1985). Maize (Rhodes et al. 1989) has also been regenerated following electroporation. The maize plants however were not fertile. Until microprojectile bombardment became available, protoplast transformation had been the major route for tranferring genes into monocots (Potrykus 1990a, 1990b). There are several drawbacks in protoplast transformation, the most important one is that regeneration of plants is often difficult. Even if plants are readily recovered from protoplasts, somaclonal variation complicates the results (Shepard et al. 1980).

Transient expression of foreign genes has been shown following fusion of protoplasts with bacterial spheroplasts

(Hain et al. 1984) and liposomes (Deshayes et al. 1985). These methods have not been as extensively reported on as have electroporation or PEG-mediated uptake.

2.3.2 MICROINJECTION

The efficiency of microinjection is high but this technique is labor intensive (Potrykus 1990a). DNA is microinjected into protoplasts, single cells, or cell clusters using micromanipulators and needles with the aid of microscopes (Neuhaus and Spangenberg 1990). Successes in both transient assays and stable transformation have been reported (Crossway et al. 1986, Reich et al. 1986, Neuhaus et al. 1987). Plants were recovered in all of these experiments; however, sexual transmission of the inserted genes has not yet been demonstrated (Neuhaus and Spangenberg 1990). Alfalfa protoplasts were transformed with a frequency of 26% (Reich et al. 1986).

2.3.3 VIRUS VECTORS

Virus vectors have been used on a limited basis for transferring genes into plant cells, and *A. tumefaciens* was used to vector the viruses. Successes have been reported using constructs of cauliflower mosaic virus (CaMV) (Brisson et al. 1984) or maize streak mosaic virus, a geminivirus

(Grimsley et al. 1986, 1987). Copies of the geminivirus genome were engineered into Ti plasmids and delivered by agroinfection into maize cells. Viruses were present in nearly all of the cells of the regenerated plants; apparently, the viruses had replicated autonomously, but stable insertion did not occur. Geminiviruses are not seed transmitted.

2.3.4 DRY SEED IMBIBITION OF DNA

Transient expression of kanamycin resistance genes have been demonstrated following dry seed imbibition of plasmid and virus vector DNA (Töpfer et al. 1989). Dry seeds of several cereals and legumes were treated with these vectors and the germinating embryos showed evidence for the resistance gene, neomycin phosphotransferase II (NPTII).

2.3.5 POLLEN TUBE PATHWAY

The pollen tube may provide a passageway for entry of DNA into plant zygotes (de la Peña et al. 1987, Luo and Wu 1988, 1989). DNA injected into the floral tiller of rye (de la Peña et al. 1987) or applied to the cut stigmatic surface of rice apparently moved into the ovary and transformed the zygote (Luo and Wu 1988, 1989). De la Peña reported a low transformation frequency of 0.07% and Luo and Wu have shown

NPTII expression in up to 20% of the seedlings from their treatments.

2.3.6 *AGROBACTERIUM*

Agrobacterium-mediated transformation in dicots has been the most widely used method for moving cassettes of genes into plant cells (Klee et al. 1987). In contrast, a handful of monocots have been stably transformed with the bacterium (Hernalsteens et al. 1984, Hooykaas-VanSlogteren et al. 1984, Schäfer et al. 1987, Grimsley et al. 1986, 1987, Raineri et al. 1990). The apparent problem with monocots is that they do not produce bacteria-inducing phenolic compounds (Usami et al. 1988).

Agrobacterium tumefaciens and the related *A. rhizogenes* are gram-negative, soil-borne facultative bacterial parasites of roots and other plant cells located at the soil line (De Cleene and De Ley 1976). Two other taxa, *A. radiobacter* and *A. rubi* are nonpathogenic and are not used in transformation (Kersters and De Ley 1984).

Agrobacterium species are natural genetic engineers that are able to insert genes present on the large (~200 bp) Ti (tumor-inducing) plasmid into the genomes of plant cells (van Larebeke et al. 1974, Watson et al. 1975, Chilton et al. 1977). A portion of this plasmid, only one of which is present in each *Agrobacterium* cell, is replicated, precisely

excised, delivered into wounded plant cells, and ligated into the nuclear genome of the invaded plant cell (Chilton et al. 1977, Chilton et al. 1980, Willmitzer et al. 1980). The mechanism by which the bacterium accomplishes this has not been fully elucidated; however, a large body of knowledge has been accumulated on Ti and Ri plasmid genes as a result of intense activity in this field (Tepfer 1989, Ream 1989).

Agrobacterium tumefaciens, the causal organism for crown galls in plants, was discovered by Smith and Townsend in 1907, and the bacteria belong to the same family as *Rhizobium*, the nitrogen-fixing bacterium in legumes (Kersters and De Ley 1984). In 1947, Braun reported that crown galls cultured on nutrient media continued to grow even though the bacteria were no longer present and phytohormones were not supplied (Braun 1958). In the 1960's, intense studies commenced on *Agrobacterium* because the galls resembled cancer tumors (Butcher 1973, Zambryski 1983a). Morel (1971) discovered opines in galls. These novel arginine derivatives are found only in crown galls and hairy roots and they are specific to strains of *Agrobacterium* (Bomhoff et al. 1976, Schroder et al. 1981). Opines were never isolated from pure cultures of *Agrobacterium* nor from uninfected plant tissues until Christou et al. (1986) reported that opines were synthesized from arginine in uninfected callus cultures.

Morel postulated that something changed in the plant genome after *A. tumefaciens* infected plants and caused gall formation. Kerr (1969, 1971) observed that virulence could be transferred from virulent to nonvirulent strains of *Agrobacterium* and to the related *Rhizobium*. He reasoned that the transfer was nonchromosomal, similar to *E. coli* plasmid exchange during conjugation. Hamilton and Fall (1971) found that *Agrobacterium* heated to 37°C lost virulence as if some cell factor disappeared, and this evidence added credence to the idea of a mobile unit. Van Montagu and Schell (1979) discovered that the large Ti plasmids are resident only in virulent *A. tumefaciens* strains and deduced that the mobile element is the plasmid. Virulent strains cured by heat treatment do not contain the plasmid. Schilperoort et al. (1979) identified the opine catabolism genes on the Ti plasmid and also observed that opines stimulate bacterial conjugation.

Once it was established that Ti plasmids are responsible for gene transfer, more information was needed about the plasmids. Maps were constructed by transposon mutagenesis (Ooms et al. 1980, Garfinkel et al. 1981). Transfer occurs despite mutation of the entire T-DNA region, and it was deduced that any gene placed into the T-DNA region could be transferred. An important portion of the Ti plasmid for T-DNA tranfer is the *vir* region that apparently

controls virulence (Klee et al. 1983, Stachel and Nester 1986).

Up to 1983, stable insertion of foreign genes into plant cells was not successful (Klee and Rogers 1989). Foreign genes had been placed into the T-DNA, they were found to be transferred into the plant genome, but no expression occurred because the coding regions of the foreign genes were ligated to bacterial rather than plant promoters. Substitution of eukaryotic genes, for example, yeast alcohol dehydrogenase, ß-globin, interferon, and SV40 early promoter genes did not yield better results. Plant gene promoters available at the time belonged to highly abundant, highly regulated genes like storage protein or leghaemoglobin genes.

Protoplast uptake of T-DNA, using the *Agrobacterium nos* gene promoter and terminator in a construct, finally resulted in expression of a chimeric gene for the bacterial enzyme chloramphenicol acetyl transferase, CAT (Paszkowski et al. 1984). CAT activity was easily assayed by a radioactive ^{32}P transfer test. Protoplast co-cultivation with intact *A. tumefaciens* resulted in transgenic calli (Marton et al. 1979, Wullems et al. 1981, Horsch et al. 1984). The first transgenic plants were regenerated following infection of tobacco cells with disarmed strains of *A. tumefaciens* in 1983, but the plasmids were not engineered with foreign genes (Zambryski et al. 1983b,

Barton et al. 1983). Stable expression of a foreign gene in tobacco callus was first reported for aminoglycoside phosphotransferase (APHII), a bacterial enzyme that confers kanamycin resistance to plant cells. It was inserted by co-cultivation of tobacco protoplasts with Ti plasmids (Fraley et al. 1983). In 1984, Horsch et al. and De Block et al. reported the first transgenic tobacco plants expressing foreign genes from *A. tumefaciens*/protoplast co-cultivation experiments.

Agrobacterium is characterized by strains of wide and narrow host range that can induce tumor formation in plants (*A. tumefaciens*) or promote excessive growth and branching of roots (*A. rhizogenes*). Both physiological responses are due to the presence of certain genes in the plasmid T-DNA. *A. tumefaciens* T-DNA contains genes that can direct phytohormone synthesis, tryptophan monooxygenase, *iaa*M and indolacetamide hydrolase, *iaa*H function in a bacterial pathway for IAA synthesis from tryptophan (Garfinkel et al. 1981, Joos et al. 1983a, Leemans et al. 1982, Willmitzer et al. 1982, Willmitzer et al. 1983, Thomashow et al. 1984, 1986). Cytokinin biosynthesis is fostered by the isopentenyl transferase gene, *ipt* (Akiyoshi et al. 1984, Barry et al. 1984). The *A. rhizogenes rol*A, *rol*B, *rol*C, and *rol*D genes are not the same as the phytohormone-synthesizing genes of *A. tumefaciens*, although *rol*D is similar in function to *iaa*M and *iaa*H (Filetici et al. 1987, Huffman et

al. 1984, Koplow et al. 1984, Lahners et al. 1984). Instead of causing tumors, *A. rhizogenes* T-DNA induces abnormal root morphology in transgenic cells, and occasionally such roots regenerate shoots (Tepfer 1984, Slightom et al. 1986, White et al. 1985). The morphology of the shoots is altered from that of the host plant. The transgenic shoots contain the T-DNA coding for the *rol*D gene (Ackerman 1977, Chilton et al. 1982, Spano and Constantino 1982, Tepfer 1984).

T-DNA has two distinct portions that are important for the bacteria, the set of plant morphology-altering genes and opine synthesis genes and the border regions. The 23-25 bp nearly perfect direct repeats are positioned at the left and right borders of all T-DNAs (Zambryski et al. 1980, Zambryski et al. 1982, Yadav et al. 1982, Barker et al. 1983, Wang et al. 1984, Slightom et al. 1986, Jouanin et al. 1989). Deletion analysis has shown that these sequences alone are responsible for the precise excision of the T-DNA between the sequences and that efficient transfer requires the 23-bp right border repeat in *cis* (Joos et al. 1983b, Shaw et al. 1984, Wang et al. 1984, Hepburn and White 1985). Border sequences can be replaced by bacterial mobilization (*mob*) and origin of transfer (*ori*T) genes (Buchanan-Wollaston et al. 1987). These genes can be substituted with broad host-range *ori*T function and high transfer function genes for efficient transformation.

Outside of the T-DNA is another important region, the *vir* region consisting of 24 genes in eight operons in the nopaline Ti plasmid. The *vir* region is inducible by plant phenolic compounds (Rogowsky et al. 1987, Stachel et al. 1985, Bolton et al. 1986, Stachel et al. 1986). Mutational analysis of the region outside the T-DNA showed that the *vir* region was required for virulence and T-DNA tranfer. The *vir* region is responsible for signal transduction, and the *vir*A and *vir*G gene products are involved in the regulation of the other operons (Ream 1989). Plant cell wounds produce lignin precursors like acetosyringone that are used as signals for T-DNA transfer by *Agrobacterium* (Bolton et al. 1986). Signal compounds are detected by the *vir*A sensor gene product that has a hydrophobic N-terminus protruding into the bacterial cytoplasm. The rest of the protein is believed to span the bacterial membrane while the C-terminus has equal numbers of hydrophobic and hydrophilic amino acids (Stachel et al. 1986, Bolton et al, 1986). The *vir*A gene product is a kinase that phosphorylates the *vir*G protein. The *vir*A/*vir*G pair comprises a two component regulatory system based on phosphorylation as communication (Ream 1989). The other *vir* genes are involved in a cascade of reactions activating various attachment, mobilization, and transfer genes that prepare the T-DNA for transfer into plant cells (Ream 1989). Phosphorylated *vir*G protein directs synthesis of a DNA-binding protein that binds to the

*vir*E promoter and also affects *vir*C and *vir*D (Toro et al. 1988). *Agrobacterium* responds to these messages by making a copy of its T-DNA. The *vir*D gene is responsible for producing the transferase that directs the synthesis of a copy of the T-DNA between the two borders (Yanofsky et al. 1986) so that the product to be transferred is excised precisely within those two points. *Vir*D encodes a topoisomerase and endonuclease that nicks and directs replication of T-DNA. T-DNA duplication and excision is believed to commence at the right border and terminate at the left (Ream 1989). Without the interest in *Agrobacterium* as a vector for gene transfer, it is doubtful that the plasmids' intricate regulatory systems would be discovered.

Also significant for plant cell transformation are two bacterial chromosome genes, *chv*A and *chv*B (Douglas et al. 1985) that direct bacterial attachment to the plant cell wall.

Agrobacterium-mediated transformation is believed to occur by the following processes:

1. Wound compounds stimulate virulence genes, two sets of genes are important, one set is on the bacterial chromosome (*chv*), one set is on the Ti plasmid (*vir*). *Agrobacterium* attaches to a target cell surface, this action is under bacterial chromosome control, its Ti plasmid *vir* genes signal site-specific endonuclease activity to cleave the

ends of the T-DNA, 5′ to 3′. Single-stranded copies are made of the bottom strand of the T-DNA.

2. T-DNA or T-strand is mobilized (transferred) from the plasmid with the aid of *mob* genes encoded by the *vir* region.

3. T-DNA is transferred across the cell walls of the bacterium and into the wounded plant cell nucleus.

4. It is integrated usually unaltered into plant nuclear DNA where transferred genes of the T-DNA are able to function because they are under the control of eukaryotic gene promoters and terminators. Examples are the *nos* promoter of nopaline synthase and *ocs* promoter of octopine synthase.

Two types of *A. tumefaciens* strains are available for plant transformation, armed and disarmed strains (Klee et al. 1987). Armed strains contain native T-DNA of Ti plasmids; plants transformed with armed strains are likely to form tumors. Disarmed strains have had the phytohormone synthesis genes removed. The disarmed strains are used almost exclusively for transformation (Klee et al. 1987). The first disarmed strain to be used was LBA4404 (Ooms et al. 1981), an octopine-type mutant that had lost all of its T-DNA. It had no antibiotic selection gene for counter selection after conjugation; thus, selection had to be done on minimal medium that prevented *E. coli* growth (Klee et al. 1987).

Other available *Agrobacterium* strains were engineered by homologous recombination to delete all or a portion of the T-DNA (Klee and Rogers 1989). One of the early constructs, pGV3850, had had all of the central T-DNA removed from Ti plasmid pTiC58 while the left and right borders and the nopaline synthase gene were intact (Zambryski et al. 1983a). In place of the deleted region, sequences from plasmid pBR322 were inserted for cointegration by homologous recombination. A large portion of the early DNA cloning had been accomplished with pBR322. The early transformation vector pGV3850 had a problem; it carried a natural resistance gene for carbenicillin (ß-lactamase gene) that precluded use of that antibiotic for killing *Agrobacterium* after co-cultivation with plant cells. Since cointegrating vectors become incorporated in the resident Ti plasmid, they cannot replicate autonomously (Klee et al. 1987).

A different construct, the split end vector pGV3111 (Fraley et al. 1985) was designed without the T-DNA and right border. The remaining T-DNA containing the left border is called the left internal homology, LIH. The target genes were introduced into *A. tumefaciens* by triparental mating, freeze/thaw, or transformation and the genes became cointegrated by homologous recombination with the LIH region. The whole target plasmid became incorporated into the cointegrate. Cointegrate vectors are

more difficult to produce than are binary vectors because conjugation frequencies are low (Klee and Rogers 1989). For example, frequencies of 10^{-1} can be obtained for binary vectors and of 10^{-4} for cointegrate vectors (Klee et al. 1987). Once generated, cointegrate vectors are easier to maintain because they need not be grown on selection media like the *Agrobacterium* strains hosting binary constructs.

Binary constructs were designed because they are easier to produce than are cointegrates. The genes to be transferred into plant cells are engineered in a binary vector, and the plasmid is placed into *Agrobacterium* where it can replicate autonomously since it does not become part of the Ti plasmid. Binary vectors can be mated or transformed into any *Agrobacterium* strain that has a resident Ti plasmid (Klee et al. 1987). The typical binary vector construct contains a wide host range origin of replication, usually from *E. coli* plasmid RK2 (Schmidhauser and Helinski 1985) and transfer genes, *trf*A. The *trf* genes can be housed on the resident Ti plasmid for maximal compactness of the binary vector. A second plasmid replication origin, usually a ColE1 replicon, is also included that functions only in *E. coli* for increasing copy number 10 to 100 times. This sequence helps for large scale plasmid preparation in probe isolation. A nopaline synthase gene is retained on some binary vectors as a scorable marker (Klee and Rogers 1989). Multilinkers for insertion of

target genes are placed between selection and scorable marker genes. The binary vector is mobilized into plant cells by signals on the disarmed host Ti plasmid.

Although armed strains of *Agrobacterium* can be used in binary vector designs, disarmed strains are preferred because their use eliminates the need to screen for isolates containing only the desired construct. The phytohormone genes as well as the right border sequence required for excision of T-DNA have been removed from binary host Ti plasmids. Triparental mating between the *E. coli* strain housing the engineered vector, an intermediate *E. coli* strain that can mate with *Agrobacterium*, and the *Agrobacterium* host results in transfer of the engineered target genes into the *Agrobacterium*.

Armed Ti-plasmids have been used in binary constructs for transformation when disarmed strains were unable to infect certain plant species (Klee et al. 1987). Tumors are produced in some transgenic plants that result from the presence of both T-DNAs being transferred into recipient plant cells. In some instances, only the desired T-DNA gets inserted (de Framond et al. 1986). Antibiotic selection eliminates tumor-only transformants while the engineered, non-tumor lines can be visually selected in most cases (Klee et al. 1987).

Choice of selectable marker genes is based on several criteria. The selective agent should be phytostatic rather

than phytotoxic in order to avoid accumulation of cell lysates from dying cells (Klee et al. 1987, Klee and Rogers 1989). Transgenic cells must be able to grow and develop into plants in the presence of the selective agent. Kanamycin resistance is by far the most widely used selectable marker (Klee et al. 1987, Klee and Rogers 1989). Neomycin phosphotransferase, type II (NPTII), is encoded by the *kan* gene that allows transformed cells to be resistant to the antibiotic kanamycin. The antibiotic binds with the 23S subunit of bacterial ribosomes, thus interfering with translation (Lehninger 1975). Resistance to the antibiotic is fostered by NPTII that phosphorylates and inactivates the aminoglycoside antibiotics (Klee et al. 1987). The *kan* gene was isolated from the *E. coli* transposon *Tn*5 (Beck et al. 1982). Selection for aminoglycoside resistance is more stringent with the compound G418; it has been used in plant species when natural resistance to kanamycin appeared to be high (Klee et al. 1987).

In chimeric constructs, the *kan* coding sequence has been fused to a strong promoter, e.g., CaMV 35S or nopaline synthase *nos* promoter (Fraley et al. 1985). Many plant transformation vectors use the 35S construct (An et al. 1985, Klee et al. 1985, Bevan 1984). Kanamycin selection is reported to be "leaky", resulting in large numbers of nontransformants or "escapes" (Klee et al. 1987). Nevertheless, numerous transgenic plants have been isolated

by kanamycin selection. If the selection pressure is too harsh, transgenic isolates may not be recovered (Klee et al. 1987). Kanamycin resistance is visually obvious as green, healthy-looking isolates, while sensitivity is characterized by growth inhibition and bleached tissues.

Other selectable markers are available. Dihydrofolate reductase (DHFR) resistance to methotrexate is conferred by a mutant form of DHFR with less affinity for the toxin (Simonsen and Levison 1983). Hygromycin and gentamicin are other antibiotics for which bacterial resistance genes have been engineered into chimeric constructs designed for expression in plant cells (van den Elzen et al. 1985, Waldron et al. 1985, Hayford et al. 1988). Herbicide tolerance has been used in several cases, for example, resistance to bromoxymil was obtained from the *Klebsiella* gene for nitrilase that detoxifies the herbicide (Stalker et al. 1988). Resistance to glyphosate (Comai et al. 1985, Shah et al. 1986) is conferred by a mutant 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase gene that codes for a form of the enzyme with 50 times lower affinity for the herbicide. Phosphinothricin resistance (De Block et al. 1987) has been used to select transgenic soybean callus (Christou et al. 1988) and maize (Gordon-Kamm et al. 1990, Fromm et al. 1990).

Scorable markers or reporter genes help to identify transgenic lines as well as distinguish transgenic plants

from "escapes". The "leakiness" of kanamycin-based selection makes the presence of a second, scorable marker useful. The most convenient and frequently used scorable marker at present is ß-glucuronidase or GUS (Jefferson et al. 1986), because of the quick and simple histochemical assay available (Jefferson 1987). GUS cleaves the substrate, 5-bromo-4-chloro-3-indoxyl-ß-glucuronide. The resulting dark blue precipitate is localized in plant cells transformed with the marker gene. Tissues must be injured prior to assaying since the substrate is not readily taken up by differentiated tissues like leaves (Jefferson 1987). Visual scoring of sliced leaves is rapid, although sometimes chlorophyll must be removed by soaking leaves in several changes of ethanol before the precipitate can be seen. A fluorometric assay whose substrate is 4-methyl-umbelliferyl-glucuronide is used as well for quantizing the GUS response (Jefferson 1987). Untransformed controls of analogous tissues must be assayed because intrinsic GUS activity has been detected in several plant species (Hu et al. 1990). Assays are often carried out under axenic conditions since bacterial contamination can result in false positives (Jefferson 1987).

Enzyme assays for the selection genes make them double as scorable markers. Several assays are based on the ability of the chimeric enzymes to phosphorylate or acetylate the selection agent. Labelled phosphate or

acetate is reacted with extracts from the putative transgenic plants and autoradiograms show the reaction product of the chimeric enzyme (McDonnell et al. 1987, Reiss et al. 1984, De Block et al. 1987). Assays for NPTII are based on transfer of labelled ^{32}P to substrate antibiotic, for example neomycin, in a dot blot assay (McDonnell et al. 1987) or in a chromatographic assay by Reiss et al. (1984).

In earlier *Agrobacterium*-mediated transformation experiments, the opines octopine and nopaline served as native markers from armed or some disarmed Ti plasids. They were assayed for using paper electrophoresis (Otten and Schilperoort 1978). Wounded plant cells sometimes produce products that give positive responses in these assays, and controls were monitored as well (Christou et al. 1986, Klee et al. 1987).

Expression cassettes have been designed for the convenient insertion of the coding regions of desired genes. The basic design consists of a strong promoter, polylinker site, and 3′ termination sequences that allow gene expression in plant cells (Velten and Schell 1985, Rogers et al. 1987). Plasmid constructs were designed containing these expression cassettes positioned between flanking chimeric genes for the selection marker NPTII and the GUS reporter gene (H. Quemada and J. Slightom, unpublished data). Cosmid vectors are also used that have a *cos* site

from bacteriophage lambda cohesive ends to allow for in vitro packaging of large inserts (An 1986).

The basic components of *Agrobacterium* transformation vectors are as follows:

1. Ti plasmid, preferably disarmed, either in a cointegrate or binary construction with target genes.
2. Border sequences or substitute sequences that are required for excision and transfer.
3. *vir* region for inducing the *Agrobacterium* to mobilize T-DNA for transfer.
4. Selection genes for bacterium and plant cell selections.
5. Plasmid origins of replication for efficiency of replication in *E. coli*.
6. Origins of conjugal transfer for mobilization into *Agrobacterium*.
7. Scorable markers for confirmation of transformation in the plant.
8. In addition, there is an optional sequence, "overdrive", in octopine-type plasmids, that increase the efficiency of endonuclease cleavage and transformation (Peralta et al. 1986).

Agrobacterium-mediated transformation is simple once a strain is found that can effectively infect a particular plant species, and the genes of interest are cloned into the vector. The other major consideration is ability to regenerate a transformed plant. Several methods for

infecting plant cells have proven effective. They rely on two types of tissues, differentiated and undifferentiated. The Horsch et al. (1985) leaf disk method is applicable in instances where differentiated tissues can either produce shoots directly or indirectly through a callus intermediate. Many dicots will dedifferentiate after treatment with growth regulators and then differentiate upon removal or manipulation of compounds (Flick et al. 1983). The leaf disk method has been used for a large number of dicot species. Another method that utilizes differentiated tissues is that of McGranahan et al. (1988) who co-cultivated somatic embryos of walnut with *A. tumefaciens*. They allowed the embryos to undergo several rounds of secondary embryogenesis on selective medium to attempt to enrich their cultures for totally transgenic isolates. Apparently, they could forego the dedifferentiation phase, a good strategy where somaclonal variation is not desired. In other instances, especially prior to the development of the leaf disk method, dedifferentiated tissues or protoplasts were co-cultivated with *Agrobacterium*, and they were successfully cultured to yield the first transgenic plants (Horsch et al. 1984, De Block et al. 1984).

Once *Agrobacterium* infects and transforms plant cells, usually two to four days are sufficient, the bacteria must be killed because they can overgrow plant tissue culture media and eventually kill the cultures (Klee et al. 1987).

Different strains of bacteria have been engineered with plasmids containing several antibiotic resistance genes; therefore, care must be taken to select antibiotics to decontaminate tissue cultures of *Agrobacterium*. Carbenicillin and cefotaxime, two highly effective compounds, are the most frequently used compounds for killing the *Agrobacterium*. Concentrations of 1.2 mM and 0.4 mM, respectively, are used (An et al. 1988). Cultures are transferred monthly since the bacteriocides and the selective agents breakdown with time.

Once putative transgenic sectors are observed on selection plates, they are rescued and either cultured continuously on selection medium, or they are removed to nonselective media where growth is usually rapid. Confirmation assays are performed to verify transformation.

2.3.7 PARTICLE GUN

The "biolistics" (biological ballistics) device or particle gun (Sanford et al. 1987) and the analogous electric discharge device (McCabe et al. 1988) may overcome many of the limitations encumbering other transformation protocols discussed earlier. Potentially any organelle, cell, tissue, or plant species can serve as a target (Sanford 1988). Transformation by delivering microprojectiles coated with plasmid DNA into intact plant

cells has been attempted in many crops in the seven years since the invention of the device (Sanford 1990). The complexity and elegance of *Agrobacterium*-mediated transformation with its cascade of *vir* gene reactions to induce T-DNA insertion into plant chromosomes are contrasted with the simple, direct force of cell bombardment with DNA-coated microprojectiles. The most remarkable thing is that bombardment seems to work quite well.

Differentiated plant tissues like leaves (Tomes et al. 1990), shoot apices (McCabe et al. 1988, Christou et al. 1989), immature zygotic embryos (Christou et al. 1988), pollen (Twell et al. 1989), somatic embryos and embryogenic calli (Gordon-Kamm et al. 1990, Finer and McMullen 1990) as well as nonembryogenic cells (Klein et al. 1988a), chloroplasts (Boynton et al. 1988), and mitochondria (Johnston et al. 1988) have been stably transformed following particle bombardment.

The most stringent proof of genome transformation is the production of transformed progeny as evidenced in the work with soybean (McCabe et al. 1988, Christou et al. 1989), maize (Gordon-Kamm et al. 1990, Fromm et al. 1990), and tobacco (Klein et al. 1988c, Tomes et al. 1990).

Evidence of transient gene expression after particle bombardment, assessed with the reporter gene for chlorampenicol acetyltransferase (CAT), was presented in the earliest studies using onion epidermal cells (Klein et al.

1987), nonregenerable maize cell cultures (Klein et al. 1988b), soybean, einkorn wheat, rice (Wang et al. 1988), and barley cell cultures (Kartha et al. 1989). The reporter gene for GUS replaced CAT as a simpler tool for quick transient assays that could be accomplished a few days after particle bombardment on very small quantities of tissues. Transient GUS expression had been detected by Wang et al. (1988) in their cell cultures as well as in barley (Mendel et al. 1989), maize, rice, and wheat (Oard et al. 1990), and in maize cells and scutellum tissues (Klein et al. 1988b).

Expression of GUS and the selection gene NPTII, has been demonstrated in transgenic plants regenerated from soybean (McCabe et al. 1988), maize (Gordon-Kamm et al. 1990, Fromm et al. 1990), tobacco (Klein et al. 1988c, Tomes et al. 1990), and cotton (Finer and McMullen 1990). Efficiency values have been reported for both transient and stable expression of reporter and selection genes, and the values have been compared to try to find a relationship between them. Frequency values for transient expression in rice, einkorn wheat, and soybean were reported to be 1/1000 (Wang et al. 1988) and 1/5000 cells for stable expression of GUS in maize calli (Klein et al. 1989). Finer and McMullen (1990) reported a stable-to-transient ratio of 0.7%. Christou et al. (1988) reported a transformation frequency of 1/100,000 cells of immature soybean embryos that were bombarded with NPTII genes and subsequently made into

protoplasts, while Oard et al. (1989) reported an efficiency of transient expression of 4.4×10^{-3} or about 1 transgenic cell in 200, similar to the results from Wang et al. (1988) and Klein et al. (1988b). These figures are comparable to transformation frequency by electroporation (Fromm et al. 1986, Lörz et al. 1985)

Lack of efficient tissue culture regeneration systems are probably not limiting factors in microprojectile bombardment transformation since targeted soybean seedling apices had been micropropagated, and among the numerous shoots recovered, some were chimeric for transgenic sectors. These chimeric sectors produced transgenic progeny (McCabe et al. 1988, Christou et al. 1989). The step requiring the largest amount of empirical experimentation may be in determining the tissues most suitable to target for efficient recovery of transformed isolates. In general, the protocol requires tissues similar to those suitable for *Agrobacterium*-mediated transformation, that is, recipient tissues for the DNA-coated particles that can develop into plants with transgenic reproductive tissues. The advantages of particle bombardment over the *Agrobacterium* method are several. Host range specificity is eliminated. All plant species, including lower plants and even unicellular organisms can, in theory, be transformed (Sanford 1990). The need to screen large numbers of plant and bacterium lines for the proper combination for efficient

transformation is thus eliminated. Following gene tranfer, the task of decontaminating cultures of *Agrobacterium* is not required and the time from gene transfer to isolation of transgenic embryos or shoots may be shorter with the bombardment technique. The unknown factors in particle bombardment reside in the transgenic product, copy number of inserted genes may be higher than delivered by *Agrobacterium* but should be comparable to results from direct uptake protocols (Klein et al. 1988a). Rearrangements of DNA have been reported and are comparable to direct uptake methods (Klein et al. 1988a).

2.3.8 COMPARISON OF PARTICLE GUN AND AGROINFECTION

Creissen et al. (1990) reported on a comparative study between agroinfection and particle bombardment of barley microspore-derived cells. The comparison could be accomplished because they used *A. rhizogenes* to deliver wheat dwarf virus (WDV) nucleic acid into barley without having to accomplish gene insertion into the barley genome. The particle gun results showed higher transformation rates, apparently because the agroinfection process was not efficient. In contrast, others have reported high success in transferring the wheat dwarf virus vector into other grasses via agroinfection (Marks et al. 1989, Boulton et al. 1989). Efficient agroinfection depended on the

Agrobacterium strain used, a problem circumvented by microprojectile bombardment.

2.4 TRANSFORMATION ASSAYS

Selection gene assays are fairly simple. Several, for example, NPTII chromatography and dot blots, require labelled compounds to be detected by autoradiography (Reiss et al. 1984, McDonnell et al. 1987). Kanamycin resistance can be determined by bioassays such as callus growth in the presence of the compound (Klee et al. 1987). An ELISA test kit for the NPTII enzyme is available (5′-3′, West Chester, PA).

The GUS histochemical assay (Jefferson 1987) is very quick and simple and can indicate the presence of chimeric plants. It is deceiving in that expression varies with age and type of tissue, even among transgenic isolates transformed with the same constructs (Benfey and Chua 1989). GUS is known to give false positive results (Hu et al. 1990). Therefore, it is a good strategy to screen for a combination of scorable markers, for example, NPTII and GUS, to confirm transformation (Klee et al. 1987).

The polymerase chain reaction (PCR, Saiki et al. 1988) has been used as a quick method to assay cells for the presence of DNA sequences complementary to the primers used. Nanogram quantities of relatively crude DNA preparations can

be amplified by PCR for sequence confirmation. Following PCR, the DNA can be blotted onto filters and hybridized with specific gene probes (Southern 1975) to demonstrate complementarity of the probes with amplification products. Southern hybridizations of genomic DNA, restricted with enzymes to give known fragment sizes, indicate genomic insertion of transferred sequences. Copy number can be estimated from Southern blots if the genome size of the plant is known. PCR and Southern data are particularly valuable in situations where expression levels are low or nonexistent. Northern blots (Maniatis et al. 1982) for detecting mRNA transcripts can indicate the presence of a gene product intermediate even if the product is not detectable. If the messenger is missing, lack of expression at the gene product level is clarified. Finally, Western blotting (Maniatis et al. 1982) can give definitive proof of the presence of the gene product from size and antibody reaction data. In addition, the antibody preparation is also utilized in enzyme-linked immunosorbent assay (ELISA) of the protein extracts directly (Clark and Adams 1977).

In addition to molecular characterization of the nucleic acids and gene products, transformants are tested for proper gene function as whole plants. Large quantities of transgenic plants are micropropagated for tests under greenhouse and field conditions. For example, plants transgenic for coat protein genes are inoculated with

extracts from virulent strains of virus. Data are collected on plants in the same manner as in breeding and cross-protection studies. Seed is produced from transgenic plants after self-pollination to determine heritability of the inserted genes (Klee et al. 1987).

Until the recent development of the particle gun (Sanford 1988), *Agrobacterium*-mediated transformation was probably the most efficient system for dicots although the efficiency of transformation was fairly high following direct DNA uptake into protoplasts (Fromm et al. 1985; Paszkowski et al. 1984) and microinjection (Neuhaus et al. 1987).

2.5 TISSUE CULTURE

To bridge the two fields, tissue culture and transformation, the comment by Cocking (1986) is appropriate. He has referred to the deluge of literature in tissue culture as a "tissue culture revolution" and commented that "Plant tissue culture permeates plant biotechnology and cements together its various aspects; to a large extent the tissue culture revolution has occurred because of the needs of this new plant biotechnology".

2.5.1 HISTORICAL PERSPECTIVE

In 1902, Haberlandt, with the acuity of a visionary, predicted from cell theory that someday people would be able to regenerate a whole plant from a single cell (Krikorian and Berquam 1969). Not only did his prediction materialize with carrot suspensions (Steward et al. 1964) and very convincingly with single cells (Vasil and Hildebrandt 1965) and protoplasts of tobacco (Cocking 1960), but it is even more significant because today plants expressing genes from other organisms, including mammals (Hiatt et al. 1989, Düring et al. 1990), have been regenerated after single cell transformation. Haberlandt attempted to grow plant parts in vitro, having little success because in the wisdom of hindsight he was working with mature tissues of three monocots and having difficulty with culture contamination (Krikorian and Berquam 1969). Today, some monocot tissues are still recalcitrant to regenerate, and decontamination of tissues from field-grown plants remains challenging. Haberlandt provided the dream.

The phytohormone indole-3-acetic acid (IAA) was identified by Went, and studied in the late 1920's for its effects on plant movement (Went and Thimann 1937). The early physiology experiments in which coleoptiles and other plant tissues were treated with auxin led to application experiments in vitro (Gautheret 1937). Gautheret (1938)

observed enhanced growth of *Salix* cambial tissue in culture media that included IAA and other growth factors. Nobecourt (1937, 1938a, 1938b) and Gautheret (1939) reported growth of small carrot explants in the presence of glucose, thiamine, cysteine hydrochloride, and IAA. White repeated the work with tobacco; thus, these three researchers are credited as pioneers in the field of plant cell culture (Street 1973). Coincidentally, tissue culture and plant transformation crossed paths as early as 1943 when Braun and White initiated in vitro cultures of crown gall tissues that grew without additional media supplements and were found to be free of bacteria. That observation eventually led to the discovery of the natural genetic-engineering ability of the crown gall bacterium, *Agrobacterium tumefaciens*, that is central to progress in the transformation field (Chilton et al. 1977). In 1939, organogenesis in tissue culture was first documented when White reported that leafy shoots were observed on cultures of a tobacco hybrid. Many growth regulators with auxin-like activity have been identified. For example, 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and benzoic acid derivatives are frequently compared for efficacy in specific applications (Kamada and Harada 1979a).

In 1957, Skoog and Miller observed shoots on tobacco callus grown on media containing autoclaved herring sperm

DNA. Previously, they had shown that the DNA promoted cell division. Kinetin was isolated from yeast and found to be a purine breakdown product of DNA (Miller et al. 1955). Natural purine compounds, partly responsible for the growth-promoting effects of coconut liquid endosperm (van Overbeek et al. 1941) were determined to be zeatin and zeatin riboside (van Staden and Drewes 1975). The purine derivative compounds in this new group of plant growth regulators were called cytokinins (Skoog and Miller 1957). Frequently used purine-derivative cytokinins are kinetin, 6-benzylaminopurine (BA), and 6-(gamma,gamma-dimethylallylamino)purine (2iP). Later, cytokinin-like activity was found in a range of seemingly different compounds, for example, in diphenylurea herbicides like N-phenyl-N'-1,2,3-thidiazol-5-ylurea (thidiazuron, Kerns and Meyer 1986). Shoot organogenesis, the ability of callus to produce shoots *de novo*, was correlated with the presence of cytokinins (Skoog and Miller 1957). Shoot initiation from callus was stimulated by high cytokinin to low auxin ratios, while root growth was enhanced by low cytokinin to high auxin ratios. For many tissue cultures this simple treatment was sufficient for recovering plants from calli (Flick et al. 1983, George and Sherrington 1984).

About a year after cytokinin stimulation of organogenesis was reported, a second method for recovering plants in culture was described in two different

laboratories. Steward et al. (1958) and Reinert (1958, 1959) observed somatic embryos in cell suspension cultures of carrot *(Daucus carota)*. The growth regulator 2,4-D was found to be critical for embryogenic callus induction (Halperin and Wetherall 1964, Kamada and Harada 1979a, 1979b), and a source of reduced nitrogen in the culture medium induced somatic embryogenesis in suspensions of carrot cells (Halperin 1966). Reduced nitrogen is provided by NH_4^+ in the high salt MS medium developed by Murashige and Skoog (1962), as well as by amino acids or undefined supplements of peptides and amino acids found in yeast extract, coconut water, casein hydrolysate, and casamino acids (Murashige 1974). Glutamine is frequently used as a source of reduced nitrogen (Nitsch 1974) and is sometimes specifically required, for example, in cotton cultures (Price and Smith 1979).

2.5.2 TISSUE CULTURE MEDIA

The large number of growth regulators, tissue types, and medium formulations available for experimentation, prompted Street (1973) to comment, "There is, at present, in all plant culture work still a strong empirical element; a necessity to arrive at technical solutions by trial and error". It is true that optimal culture conditions for each plant species must be determined empirically; however,

widely used medium formulations and a large collection of published literature exists for the researcher (Street 1973, Flick et al. 1983, Ammirato 1983, George and Sherrington 1984, George et al. 1987, 1988). A simple starting point is offered by Ammirato (1983) who summarized a large body of literature and concluded that 2,4-D and a source of reduced nitrogen are the minimum supplements to media necessary for inducing somatic embryogenesis. By far, the most widely used medium is Murashige and Skoog's MS high salts medium (Torrey 1985) that contains a source of nitrate nitrogen (NO_3^-) as well as reduced nitrogen (NH_4^+), myo-inositol, nicotinic acid, pyridoxine·HCl, thiamine·HCl, sucrose, and agar at pH 5.8 (Murashige and Skoog 1962). Media usually contain the 16 essential macro- and microelements necessary for higher plant growth (C, H, O, P, K, N, S, Ca, Fe, Mg, B, Z, Cu, Mo, Mn, and Cl). The major components are provided in the form of sugar and as nitrates, phosphates, and sulfates (Yeoman 1973). Vitamins, amino acids, myo-inositol, iron chelate (Fe-EDTA), and growth regulators are often included but they are not essential for growth (Ammirato 1983). Other frequently used media are Gamborg's B5 (Gamborg et al. 1968), a high salt medium that contains reduced levels of ammonium and high levels of nitrate, N6 (Chu 1978) that is similar to B5, and White's medium (White 1963). N6 was initially formulated for anther cultures, but it is widely used for embryogenesis in the Poaceae

(Armstrong and Green 1985). White's medium is a low salt formulation with an osmotic concentration of 0.015 MPa vs. 0.19 MPa for MS, while osmotic concentrations in embryo ovular fluids have been reported to be 0.9 to 1.0 MPa (Ammirato and Steward 1971).

2.5.3 PLANT MATERIAL AND DECONTAMINATION

Possibly every tissue of the "model plants" tobacco and carrot has been placed into culture. A wide array of tissues from many species has been cultured as evidenced by the lists of Ammirato (1983), Flick et al. (1983) and Williams and Maheswaran (1986). Most often, tissues bearing meristematic cells, for example, actively growing shoot apices, root tips, petioles, hypocotyls, cotyledons, peduncles, and flower buds, have been surface-sterilized and cultured. Fairly large numbers of explants from field-grown tissues should be decontaminated since losses due to contamination can be high (Ammirato 1983). Surface sterilization is most often accomplished with diluted commercial bleach. Tissues are soaked or agitated for three to 30 minutes in 1 to 2% sodium hypochlorite containing a drop or two per liter of a surfactant like Tween 20 (Ammirato 1983). Mercuric chloride is used in concentrations of 0.1 to 1% for varying lengths of time, although the salt is highly toxic to plant and animal

tissues (Yeoman 1973). Following surface decontamination, freshly isolated explants are sometimes immersed in media containing antibiotics and fungicides to control bacteria and fungi that escape oxidation by the bleaching agent or heavy metal poisoning (Yeoman 1973). Compounds like the bacteriocide rifampicin and fungicide benomyl have been successfully utilized for decontamination of woody *Camellia sinense* and *C. japonica* cuttings (Haldeman et al. 1987).

Explant size can affect successful culture establishment. Explants have ranged in size from a few mm in length to several cm (Ammirato 1983). The smaller the explant, the larger the number of pieces available for culture. Yeoman (1973) provides examples of minimum explant sizes; for carrot root secondary phloem explants it is 3.8 mg of tissue, about 25,000 cells, whereas for Jerusalem artichoke tuber, it is 8.0 mg containing about 20,000 cells. Often, the larger the explant, the greater the chances of survival (Yeoman 1973) but the greater the risk of contamination (Ammirato 1983).

Seedlings are often germinated aseptically on media, sterile wet filter papers, or water agar after surface-sterilizing the testae. This treatment is simple and eliminates damage to delicate seedling tissues. Fresh or dried seeds are soaked in 10 to 20% (v/v) commercial bleach or in 0.1 to 1% mercuric chloride for five to 30 minutes, similar to explant tissue decontamination (Ammirato 1983).

A special modification of seed culture is embryo rescue (Laibach 1929), where immature ovules are explanted to nutrient media for various genetic or physiological experiments. Hybrids from wide crosses have been recovered by embryo rescue.

2.5.4 DIRECT AND INDIRECT PLANT REGENERATION BY ORGANOGENESIS AND SOMATIC EMBRYOGENESIS

Hundreds of plant species, even the more difficult to culture monocots, regenerate shoots either directly or following a callus phase (Flick et al. 1983, George et al. 1987, 1988), and/or they produce somatic embryos in tissue culture (Ammirato 1983, Williams and Maheswaran 1986). The direct method is preferred over the indirect in situations where minimal somaclonal variation (Larkin and Scowcroft 1981) is desired. Passage through tissue culture has been known to result in plants and cells exhibiting aberrant cytological, phenotypical, and genotypical changes (Sacristan 1971, D'Amato 1978, Orton 1985) and is assumed to be the basis of somaclonal variation (Larkin and Scowcroft 1981). The indirect method includes dedifferentiation into callus followed by differention into shoots or somatic embryos (Sharp et al. 1980, Williams and Maheswaran 1986) and is desired for cultures requiring small cell aggregates for protoplast preparation or for variant selection.

Abundant examples of direct and indirect regeneration exist in the literature where both organogenic and embryogenic cultures have been produced (Williams and Maheswaran 1986). *De novo* development of shoots from mature or immature explants is well known in horticultural crops like African violets, and crassulacean species that proliferate new shoots from leaf cuttings (Janick 1973). Next to asexual propagation by rooted cuttings, marcotting, and micropropagation, direct shoot organogenesis or embryogenesis is believed to preserve the original genotype since the sometimes disruptive callus phase is omitted (Sacristan 1971, D'Amato 1978). Direct organogenesis often does not require growth regulators (Flick et al. 1983). Williams and Maheswaran (1986) list several species, for example, *Apium graveolens, Atropa belladona, and Biota orientalis,* that produce somatic embryos rather than shoots directly from petiole, pollen, or immature embryo tissues, respectively.

Callus cultures are most often initiated with the growth regulator 2,4-D or some other auxin substitute with or without a cytokinin (Ammirato 1983). After calli are established, within weeks or months, manipulations of the auxin and cytokinin concentrations often precede recovery of shoots. Cytokinins alone have no apparent callus-inducing properties (Ammirato 1983). Calli are often induced by high concentrations of both auxins and cytokinins, about 5.0 to

50 μM of each, and shoots subsequently develop on media containing high cytokinin to low auxin ratios, for example, 0.5 μM auxin and 5.0 μM cytokinin (Flick et al. 1983).

Organogenesis has been demonstrated more frequently than has somatic embryogenesis, although potentially larger numbers of plants are produced from somatic embryogenesis than organogenesis (Ammirato 1983).

2.5.5 SOMATIC EMBRYO DEVELOPMENT

Somatic embryos sometimes cease to grow at an early stage of development and need additional treatment for maturation (Ammirato 1983, Williams and Maheswaran 1986). Abscisic acid (ABA) and cytokinins, according to Ammirato (1983), stimulate maturation of somatic embryos. Germination subsequently occurs on media either devoid of auxin or containing low concentrations of cytokinins.

2.5.6 MICROPROPAGATION

Micropropagation has become a profitable applied field due to the valuable horticultural crops, notably orchids, that are propagated for commerce (George and Sherrington 1984, Sagawa and Kunisaki 1990). The protocol involves identification of superior genotypes to mass propagate, decontamination of the field- or greenhouse-grown material,

and induction of rapid proliferation with cytokinins (Murashige 1974, Flick et al. 1983). Once shoots are produced from any of the above regeneration and propagation methods, the bulk of the task to produce a whole plant is solved; however, rooting may not be easily acommplished. Since germinating somatic embryos by definition produce roots and shoots, these propagules are fairly easy to move to the pot and field environment by as long as the plant is acclimatized at each step. However, shoots may prove recalcitrant to rooting. Most dicot shoots root well with NAA or IBA treatment (Street 1973, Flick et al. 1983). A generally useful concentration is 5.0 μM of either growth regulator, in agar-solidified or liquid medium (Street 1973). Rooted propagules are acclimitized to greenhouse and field conditions in chambers of high humidity with a gradually decreasing relative humidity (Murashige 1974).

Ammirato (1983) and Williams and Maheswaran (1986) have listed about 20 dicotyledonous families in which somatic embryos have been observed in tissue culture, Caricaceae among them.

2.6 METHODS IN PAPAYA TISSUE CULTURE

Papaya tissue cultures have been initiated for three major reasons, embryo rescue, callus production, and micropropagation. Ovules from immature fruit have been

cultured in attempts to rescue hybrid embryos before they aborted (Manshardt and Wenslaff 1989a). Callus has been generated to differentiate large numbers of shoots or plants (Litz 1984) or to produce the proteolytic enzyme papain (Medora et al. 1973, Yamamoto et al. 1986). Since papaya segregates 2:1 for sex in hermaphrodite lines and 1:1 in dioecious lines (Storey 1953), shoots from field-grown trees have been micropropagated for mass production of desired genotypes (Litz and Conover 1978a, 1978b, 1980, 1981a, Drew and Smith 1986, De Winnaar 1988, Drew 1988, Miller and Drew 1990, Reuveni et al. 1990). The following section constitutes a detailed review of papaya tissue culture techniques. The review is organized into sections representing the different components of the tissue culture process, for example, explant treatment or rooting of propagules.

2.6.1. PAPAYA OVULE CULTURES

Papaya ovules have been cultured with the intent to rescue interspecific hybrid zygotes (Khuspe et al. 1980) after the method of Laibach (1929). In papaya, wide hybridizations were successfully accomplished by Khuspe et al. (1980), Litz and Conover (1981b, 1982, 1983), Moore and Litz (1984), and Manshardt and Wenslaff (1989a, 1989b). Failure of endosperm development required embryo rescue of

hybrids (Manshardt and Wenslaff 1989a). The tissues rescued were determined to be highly embryogenic. Immature ovules were dissected from 20- to 182-day-old green *Carica* fruits that had been control-pollinated. The fruits were surface-sterilized in 0.5 to 2% sodium hypochlorite with or without a few drops of Tween 20 for ten to 30 minutes, rinsed with sterile, distilled water, and then slit open to free the ovules. Since the ovules were removed from a presumably uncontaminated chamber, the cultures resulting from ovule culture should have had minimal contamination.

2.6.2 PAPAYA EXPLANTS FROM FIELD-GROWN TREES

Field-grown tissues of papaya and other *Caricas* have been isolated either for direct micropropagation of selected genotypes, or they have been put into callus culture for various reasons (De Bruijne et al. 1974, Jordan et al. 1982). Most researchers have prepared field-grown explants ranging from 1-mm to 2-cm long by rinsing them in 70 to 100% ethanol followed by immersion in 1% (w/v) sodium hypochlorite with or without surfactant for three to 12 minutes with continuous agitation (De Bruijne et al. 1974, Litz and Conover 1980, 1978a, 1978b, 1981a, Jordan et al. 1982, Drew and Smith 1986, Drew 1988). De Winnaar (1988) used a similar decontamination treatment to establish micropropagation of 'Sunrise Solo' shoots from lateral

branches of field-grown trees and apical shoots from greenhouse plants by agitating them for ten to 15 minutes in distilled water containing a few drops of detergent prior to the bleach treatment. The surface-sterilized, rinsed tissue was trimmed of external surfaces leaving a 2 x 2-mm apex for culture. Reuveni et al. (1990) added a bacteriocide treatment to shoot explant decontamination with sodium hypochlorite. The field-grown explants were rinsed in 364.5 μM rifampicin solutions, dipped into sodium hypochlorite, citric, and ascorbic acid solutions, or they added 60.8 μM rifampicin to the incubation medium and reported good control of bacterial contamination. Decontamination of rapidly growing lateral shoots was recommended by both Reuveni et al. (1990) and Drew (1988) to optimize success. Reuveni et al. (1990), Drew (1988), and Litz and Conover (1981a) observed seasonal fluctuations in contamination rates, the lowest rates were observed with explants that were collected in cooler, drier periods when shoot growth was active.

Mercuric chloride was used by others to decontaminate explants collected from plants in the field and greenhouse. Yie and Liaw (1977) dipped 1.5- to 2-mm-long shoot tips into 70% ethanol for a few minutes before mercuric chloride treatment. Explants 1.5- to 5-mm long were placed into 0.1% mercuric chloride with or without surfactant for five to 15 minutes followed by several rinses with sterile distilled

water (Yie and Liaw 1977, Pandey and Rajeevan 1983, Rajeevan and Pandey 1986). In addition, Rajeevan and Pandey (1986) agitated the decontaminated explants in sterile water for two to three hours before placing them on nutrient media.

2.6.3 PAPAYA SEED CULTURES

Papaya cultures prepared by Arora and Singh (1978a, 1978b) consisted of 4- to 5-mm-long hypocotyl sections (about 15 mg fresh weight) from aseptically germinated four- to eight-week-old papaya seedlings. Seeds were collected from ripe fruit surface-sterilized with 0.1% mercuric chloride for 20 minutes. The seeds were removed from the sliced fruit, freed of the fleshy sarcotestae, and germinated on White's medium (White 1963) supplemented with 10.4 μM GA_3. Yie and Liaw (1977) germinated surface-sterilized seeds of 'Solo No. 1' that had been immersed in 70% ethanol for two minutes followed by ten minutes of soaking in 0.1% mercuric chloride. After several rinses with sterile distilled water, the seeds were germinated on moistened filter papers in erlenmeyer flasks in darkness. Internode sections from seedlings of unspecified age were explanted. Yamamoto et al. (1986) surface-sterilized mature papaya seeds with 1% antiformin, germinated them on 1% agar, and cultured about 1-cm-long hypocotyl explants. Seedling age was not reported. Litz et al. (1983) surface-sterilized

'Solo' papaya seeds in 2% (w/v) sodium hypochlorite containing a few drops of Tween 20 for 30 minutes and rinsed the seeds three times with sterile distilled water. Four to six seeds were germinated in the dark on moist filter paper in 100-mm petri dishes. Eleven- to 12-day-old seedling cotyledons were sliced into lamina and midrib portions and placed on media. Chen et al. (1987) rinsed 'Solo' and 'Sunrise' seeds for one hour in tap water, immersed them in 75% ethanol for one minute, and in 0.1% sodium hypochlorite for ten minutes. After several rinses with sterile distilled water, the seeds were sown on MS agar medium. Explants consisted of shoots, stems, leaves, cotyledons, and roots without root tips from four- to six-week-old seedlings with three leaves (5- to 8-cm-tall plants). Explants were 3- to 5-mm long.

2.6.4 PAPAYA TISSUE CULTURE MEDIA

Table 2.1 shows the nutrient media formulated for various types of papaya cultures. A brief summary of the results of the different treatments is included. The most widely used medium was MS. Callus cultures were most frequently induced with NAA and micropropagation was most readily accomplished with about 10 μM BA or kinetin.

Table 2.1. Media used for tissue culture of papaya

CALLUS CULTURES

SPECIES	EXPLANT	MED1	GROWTH REG1 (μM)	MED2	GROWTH REG2 (μM)	RESULTS	CITATION
Carica							
papaya	seedling petiole	MS	NAA 1 2iP 10	W	BA 0.01 NAA 0.1	Embryogenic calli	De Bruijne et al. 1974
				or W and 0.1MS	ZEA 0.001 BA 0.01 BA 0.01 NAA 0.1	Embryogenic calli Somatic embryos, no plants	
papaya	seedling stem internodes	MS	NAA 5.4 K 0.46	MS	IAA 29 K 4.6-9.3	Somatic embryos, plants, micropropagation, roots	Yie and Liaw 1977
papaya	stem sections, shoot tips	MS	K 4.6	MS	IBA 29 K 4.6	Micropropagation	Mehdi and Hogan 1976
papaya	seedling shoot tips, mature shoots, internodes, seedling internodes	MS MS	IBA IAA K NAA CW			Somatic embryos, plants, micropropagation, roots	Mehdi and Hogan 1979

Table 2.1. (Continued) Media used for tissue culture of papaya

CALLUS CULTURES

SPECIES	EXPLANT	MED1	GROWTH REG1 (μM)	MED2	GROWTH REG2 (μM)	RESULTS	CITATION
Carica							
papaya	seedling, 4-6 wk old: stem, cotyledon, root, leaf, shoot tip, 3-5 mm	½MS	NAA 5.4 K 2.3 GA 2.6			Somatic embryos and plants from root callus	Chen et al. 1987
		or ½MS	NAA 5.4				
		or ½MS	NAA 5.4 GA 2.6				
papaya	seedling, 4-8 wk old: germinated on W + GA, stem, 4-5 mm	LS	NAA 10.8 K 2.3 GA 2.6	LS	NAA 1.1 K 9.3	Shoot organogenesis	Arora and Singh 1978a
papaya	seedling, 4-6 wk old: germinated on W + GA, stem, 4-5 mm	LS	NAA 10.8 K 2.3 2,4-D 0-45 K 0-46			Callus	Arora and Singh 1978b

Table 2.1. (Continued) Media used for tissue culture of papaya

CALLUS CULTURES

SPECIES	EXPLANT	MED1	GROWTH REG1 (μM)	MED2	GROWTH REG2 (μM)	RESULTS	CITATION
Carica							
papaya	seedling, 1-cm pieces	LS	2,4-D 9	LS	2,4-D 0.09 K 0.93	Somatic embryos but plants not sought	Yamamoto and Tabata 1989
papaya	seedling 1-cm pieces	LS	2,4-D 9			Callus	Yamamoto et al. 1986
papaya	seedling, 6 wk old: stem, petiole, shoot, 5 mm	MS B5 MS	NAA 9 K 2.3 NAA 5.4 K 46	MS	NAA 0.54 BA 2.2	Callus micropropagation	Pandey and Rajeevan 1983
papaya	field-grown lateral shoots	MS	NAA .54-9 K 46	MS or MS or MS	BA 2.2 ZEA 4 2iP 7.9 NAA 0.54 BA 2.2	Micropropagation, fast short shoots	Rajeevan and Pandey 1986
papaya	seedling hypocotyl, 4 cm	WR	2,4-D 9	WR	2,4-D 1.8	Callus	Medora et al. 1973

Table 2.1. (Continued) Media used for tissue culture of papaya

CALLUS CULTURES

SPECIES	EXPLANT	MED1	GROWTH REG1 (μM)	MED2	GROWTH REG2 (μM)	RESULTS	CITATION
Carica							
papaya	seedling hypocotyl, 4 cm	WR	2,4-D 9	WR	2,4-D 1.8	Callus	Medora et al. 1979
papaya	seedling hypocotyl, 4 cm	WR	2,4-D 9	WR	2,4-D 1.8	Callus	Medora et al. 1984
papaya	seedling cotyledon midrib, lamina	½MS	NAA 2.7-27 BA 1.3-13.2	½MS	NAA 0-10.7 BA 0.22-8.9	Shoots from organo-genesis	Litz et al. 1983
papaya x *cauliflora*	ovules, 2 mo old	W		W		Embryo rescue	Khuspe et al. 1980
papaya x *cauliflora*	ovules, 20-120 d	W	CW 20% or BA 0.44-4.4			Somatic embryos but no plants	Litz and Conover 1981b
papaya x *cauliflora*	ovules, 20-40 d	W	CW 20%	W	NAA 0.54-10.7 BA 0.22-0.9	Somatic embryos, plants	Litz and Conover 1982
papaya x *cauliflora*	ovules, 20-140 d	½MS	CW 20%	½MS	2,4-D 4.5-9	Somatic embryos, plants	Litz and Conover 1983

Table 2.1. (Continued) Media used for tissue culture of papaya

CALLUS CULTURES

SPECIES	EXPLANT	MED1	GROWTH REG1 (μM)	MED2	GROWTH REG2 (μM)	RESULTS	CITATION
Carica							
papaya x *cauliflora*	ovules, 65 d	½MS	CW 20%	½MS		Somatic embryos, plants	Moore and Litz 1984
papaya x *cauliflora*	ovules, 65 d	½MS	CW 20%	½MS	2,4-D 9	Somatic embryos	Litz 1986a
papaya x *cauliflora*	ovules, 90-180 d	MS or W	CW 20%	MS	NAA 2.7 BA 0.9	Somatic embryos, plants	Manshardt and Wenslaff 1989a
papaya x *pubescens*, *quercifolia*, *stipulata*, & *pubescens* x *papaya*	ovules, 90-180 d	MS or W	CW 20%	MS	NAA 2.7 BA 0.9	Somatic embryos, plants	Manshardt and Wenslaff 1989b

Table 2.1. (Continued) Media used for tissue culture of papaya

CALLUS CULTURES

SPECIES	EXPLANT	MED1	GROWTH REG1 (μM)	MED2	GROWTH REG2 (μM)	RESULTS	CITATION
Carica							
stipulata	peduncle	MS	NAA 1 BA 2	MS or MS or MS or MS	NAA 1 BA 2 ± charcoal BA 2 NAA 1 ± charcoal	Somatic embryos, plants	Litz and Conover 1980
candamarcensis (*pubescens*)	seedling, 2 mo old: hypocotyls	NN or MS	NAA 5.4-27 K 4.5			Somatic embryos, plants	Jordan et al. 1982

MICROPROPAGATION OF PAPAYA

SPECIES	EXPLANT	MED1	GROWTH REG1 (μM)	MED2	GROWTH REG2 (μM)	RESULTS	CITATION
papaya	seedling apices, 2 mo old, apices 4 mo. old	deF	NAA 1 BA 1			Micropropagation, few roots	Drew and Smith 1986

Table 2.1. (Continued) Media used for tissue culture of papaya.

MICROPROPAGATION OF PAPAYA

SPECIES *Carica*	EXPLANT	MED1	GROWTH REG1 (μM)	MED2	GROWTH REG2 (μM)	RESULTS	CITATION
papaya	field-grown axillary rooted cuttings or buds, 6 mo old	DS	NAA 1 BA 1	DS	NAA 0.25 BA 1	Micropropagation, rooted plants to field	Drew 1988
		DS	NAA 0.5 BA 2				
papaya	shoots, 6 mo old	deF	NAA 1 BA 1	DS	NAA 0.5 BA 2	Micropropagation, rooted plants to field	Miller and Drew 1990
papaya	lateral shoots of hedged rooted cuttings or mainstem buds	MS	NAA 0.54 BA 2.2			Micropropagation, rooted plants	Reuveni et al. 1990
papaya	apices	MS	NAA 0.54-9 K 4.6-46 or IAA 3.4-11.4 BA 0.22-9	MS	NAA 0.54 BA 2.2 or NAA 10.7 K 46	Micropropagation	Litz and Conover 1977
papaya	apices, mature, field-grown	MS	NAA 10.7 K 46	MS	NAA 1.1 BA 2.2	Micropropagation	Litz and Conover 1978a

Table 2.1. (Continued) Media used for tissue culture of papaya

MICROPROPAGATION OF PAPAYA

SPECIES	EXPLANT	MED1	GROWTH REG1 (μM)	MED2	GROWTH REG2 (μM)	RESULTS	CITATION
Carica							
papaya	apices	MS	NAA 10.7 K 46	MS	NAA 0.54 BA 2.2	Microprop-agation	Litz and Conover 1978b
papaya	shoot tips, mature, field-grown	MS	NAA 10.7 K 46	MS	NAA 1.1 BA 2.2	Microprop-agation, rooted plants	Litz and Conover 1981
papaya	apices, field, nursery plants	½MT	NAA 1.1 BA 2.2	½MT	NAA 0.54 BA 2.2	Microprop-agation, rooted plants	De Winnaar 1988

Media abbreviations:

MS = Murashige and Skoog (1962)
LS = Linsmaier and Skoog (1965)
W = White (1963)
B5 = Gamborg et al. (1968)
WR = White and Risser (1964)
NN = Nitsch and Nitsch (1969)
DS = Drew and Smith (1986)
deF = de Fossard (1974)
MT = Murashige and Tucker (1969)

Table 2.1. (Continued) Media used for tissue culture of papaya

Other abbreviations:

MED1 = initiation media
MED2 = other media for further development, proliferation, etc.
GROWTH REG1 = concentrations of growth regulators for culture initiation
GROWTH REG2 = concentrations of growth regulators used in other media
NAA = 1-naphthaleneacetic acid
2iP = 6-(gamma,gamma-dimethylallyloamino)purine
K = kinetin
IBA = indole-3-butyric acid
IAA = indole-3-acetic acid
CW = coconut water
GA = gibberellic acid
BA = 6-benzylaminopurine
ZEA = zeatin
2,4-D = 2,4-dichlorophenoxyacetic acid

2.6.5 DIRECT REGENERATION OF PAPAYA: SOMATIC EMBRYOGENESIS AND ORGANOGENESIS

In the papaya tissue culture literature, direct plant regeneration without a callus phase has not been reported for papaya; however, the polyembryonic interspecific hybrid zygotes of *C. papaya* x *C. cauliflora* (Litz and Conover 1981b, 1982, 1983; Moore and Litz 1984; Manshardt and Wenslaff 1989a) and the interspecific progeny from crosses of *C. papaya* with *C. pubescens, C. quercifolia,* and *C. stipulata* yielded direct somatic embryos as well as embryogenic calli (Manshardt and Wenslaff 1989b). In some examples, the zygotic embryo divided to make several embryos without evidence of a callus phase (see photographs, Litz and Conover 1981b, 1983; Manshardt and Wenslaff 1989a). The cultures were initiated on half-strength or full-strength MS medium containing no growth regulators other than coconut water (Table 2.1).

2.6.6 INDIRECT REGENERATION OF PAPAYA: ORGANOGENESIS AND SOMATIC EMBRYOGENESIS

A majority of the examples of *de novo* papaya regeneration had ensuing callus phases. De Bruijne et al. (1974) first observed somatic embryos of *Carica papaya* on "humid" petiole calli initiated on MS medium containing

1.0 μM NAA and 10 μM 2iP. However, they were not able to continue culture of the embryos beyond the cotyledonary stage. Yie and Liaw (1977) and Mehdi and Hogan (1979) reported somatic embryos from papaya seedling stem cultures grown on MS medium containing an auxin and a cytokinin (Table 2.1). Mehdi and Hogan (1979) reported an intermediary callus phase of 'Solo No. 8' before somatic embryogenesis was observed on shoot tip and internode callus from mature plants and seedlings that were produced on MS medium containing coconut water. They reported that IAA, IBA, NAA, and kinetin supplemented their medium. The frequency of plant regeneration was not indicated in either case, although each of the two groups reported that plants were recovered.

The most convincing evidence for high frequency somatic embryogenesis in papaya cultures was presented by Chen et al. (1987) who cultured seedling root sections freed of apices on half-strength MS (1/2MS) medium containing 5.4 μM NAA, 2.3 μM kinetin, and 2.6 μM GA_3. Brown callus on root cuttings produced somatic embryos (Table 2.1). They recovered embryogenic calli after three months of culture and reported that one embryogenic root callus cluster could produce over one hundred somatic embryos. More than 40 plants were planted in the field. Photographs showed large numbers of well-developed somatic embryos with cotyledons.

Papaya hybrids appear to be highly embryogenic if cultured as zygotic embryos. Ovule cultures from papayas hybridized with other *Carica* species developed polyembryonic zygotes or embryogenic calli after three months of culture on White's, 1/2MS, or MS media and they produced large numbers of somatic embryos (Litz and Conover 1981b, 1982, 1983, Moore and Litz 1984, Manshardt and Wenslaff 1989a, 1989b) (Table 2.1).

Litz and Conover (1982, 1983) described a compact, hard callus at the micropylar end of immature ovules cultured on 1/2MS medium containing 20% CW, 6% sucrose, and 2.7 mM glutamine that later proliferated embryos on the surface of the callus. They reported indirect somatic embryogenesis from similar ovular tissues cultured earlier on the more dilute White's medium with the same results (1981b). Litz and Conover had initiated embryogenic suspension cultures with hybrid callus in 1/2MS medium containing 6% sucrose, 2.7 mM glutamine, 0.3 mM myo-inositol, 20% CW, and 4.5 μM 2,4-D (1983). Moore and Litz (1984) described ovular callus that gave rise to somatic embryos in suspension culture, and they determined by isozyme analysis that the cultures resulted from hybrids of the interspecific crosses. In addition, Litz and Conover (1980) produced highly embryogenic callus cultures of *C. stipulata* from peduncle-derived calli that had been initiated on MS medium containing NAA and BA (Table 2.1).

Jordan et al. (1982) reported embryogenic *C. candamarcensis* (synonymous with *C. pubescens*) callus from hypocotyl calli initiated on MS medium containing NAA and BA. This mountain papaya is of little commercial value, although its genes for cold resistance may be important.

Manshardt and Wenslaff (1989a) repeated the *C. papaya* x *C. cauliflora* crosses attempted by Litz and Conover (1981b) and Khuspe et al. (1980), and they produced reciprocal crosses as well. They cultured ovules of approximately the same ages as those used by Litz and Conover (1981b, 1982, 1983) and older ones, 90 to 180 days post-anthesis in age. Moore and Litz (1984) observed polyembryony as well as undifferentiated callus-like masses in some dissected ovules of the hybrids, and Manshardt and Wenslaff corroborated the findings. In addition, Manshardt and Wenslaff (1989b) recovered *C. papaya* hybrids from crosses with *C. stipulata, C. pubescens,* and *C. quercifolia* under similar culture conditions. The resulting somatic embryos were also products of polyembryony and indirect embryogenesis.

Hence, hybrid ovule cultures have given rise to both direct and indirect somatic embryogenesis using White's or 1/2MS media containing no growth regulators but supplemented with 6% sucrose, 20% CW, and 2.7 mM glutamine.

Papaya organogenesis reported in the lone experiment by Litz et al. (1983) resulted from culture of cotyledon lamina and midrib tissues. Calli developed on 1/2MS medium

containing 2.7 to 27 μM NAA and 1.3 to 8.9 μM BA, and later, shoots were regenerated after the growth regulators in the medium were decreased.

2.6.7 PAPAYA SOMATIC EMBRYO MATURATION AND GERMINATION

Papaya researchers, in general, have had little trouble germinating somatic embryos. De Bruijne et al. (1974) may have needed to treat embryos with maturation-inducing compounds like cytokinins or ABA because they were not able to grow somatic embryos into plants. Other groups recovered plants after somatic embryos were transferred from induction media to media devoid of growth regulators or containing low concentrations of auxin and cytokinin.

Somatic embryos of *C. stipulata* germinated on MS medium alone (Litz and Conover 1980). Jordan et al. (1982) recovered germinated plants after transferring somatic embryos of *C. candamarcensis* to 1/2MS medium containing 2.6 μM IAA and 0.2% casein hydrolysate. Litz and Conover (1982) germinated somatic embryos from interspecific hybrids on White's medium containing 0.5 to 10.7 μM NAA and 0.2 to 8.9 μM BA. In other reports, interspecific hybrid embryos developed normally on 1/2MS medium containing CW, glutamine, and 6% sucrose (Litz and Conover 1983) or on the same medium containing 3% sucrose (Litz 1986a). Higher concentrations

of BA caused excessive swelling of hypocotyls and inhibited shoot and leaf development.

Manshardt and Wenslaff (1989a) germinated interspecific somatic embryos on MS medium with or without 0.1% casein hydrolysate. Most of their embryos did not germinate normally, but treatment with medium containing 2.7 μM NAA and 8.9 μM BA stimulated germination. Roots formed within two weeks. Chen et al. (1987) germinated somatic embryos on 1/2MS medium containing 5.4 μM NAA, 2.3 μM kinetin, and 2.6 μM GA_3. Germinated embryos were transferred to MS medium containing 5.4 μM NAA, and plants that rooted on this medium were ready for potting in soil. Yie and Liaw (1977) germinated somatic embryos in 15 days in liquid or agar-gelled Linsmaier and Skoog medium (LS, Linsmaier and Skoog 1965) containing 15% CW, 3% sucrose, and 28.5 μM IAA.

2.6.8 PAPAYA SHOOT DEVELOPMENT

Arora and Singh (1978b) apparently obtained shoots via organogenesis from stem callus on LS medium containing 1.1 μM NAA and 9.3 μM kinetin. It is not clear if the shoots arose from nodal buds on the seedling stem sections or developed *de novo*. The former response would be a form of micropropagation. The only other example of organogenesis in papaya tissue cultures is that of Litz et

al. (1983) who reported shoots arising from cotyledon lamina and midrib calli (Table 2.1).

2.6.9 PAPAYA MICROPROPAGATION

Shoot tip micropropagation from field-grown explants was apparently simple once the tissues were decontaminated. Mehdi and Hogan (1976, 1979), Yie and Liaw (1977), Litz and Conover (1978a, 1978b), Pandey and Rajeevan (1983), Rajeevan and Pandey (1986), Litz (1986b), Drew (1988), De Winnaar (1988), and Reuveni et al. (1990) provided ample evidence that papaya micropropagation was possible. Each used some form of cytokinin to increase numbers of shoots from an initial explant. The media used in each report are listed in Table 2.1. In general, MS medium supplemented with fairly low concentrations of cytokinin, for example, about 10 μM kinetin or BA, appears to be effective for papaya shoot micropropagation. The technique may benefit papaya growers especially since the seeds of hermaphrodites segregate 2:1 for sex (Storey 1953). For two hermaphrodites, there is one female. The homozygous male is apparently a lethal gene combination (Storey 1953). Farmers plant several seeds in a mound and thin seedlings once sex is determined after four to six months (Storey 1953). If micropropagated shoots can be economically produced, such a practice could be eliminated.

2.6.10 ROOTING, ACCLIMATIZATION, TRANSFER OF PAPAYAS TO GREENHOUSE AND FIELD

Papaya shoots have been induced to root in MS medium containing 2.7 μM NAA or 4.9 μM IBA (Litz and Conover 1981) after ranges of 1.1 to 5.4 μM NAA and 4.9 to 14.8 μM IBA were tested (Litz and Conover 1978a). Litz and Conover (1981) acclimated rooted plants in peat:perlite mixtures under intermittent misting. Drew (1988) rooted 1-cm-tall shoots in Drew and Smith medium (DS, 1986) supplemented with 9.8 μM IBA. Miller and Drew (1990) determined that shoot length was important for root induction and development. Shoots less than 5-mm long rooted in only 35% of the cultures after 45 days, whereas in seven days nearly 65% of the 1.6- to 2.0-cm long shoots rooted, and by 18 days 80% of them had roots. Shoots 1-cm long were only a little lower in percentage of rooting and slower; therefore, the optimum size of cuttings for rooting appeared to be 1- to 2-cm long. If shoot stems were too short for root initiation, GA_3 has been used to help elongation (Chen et al. 1987); however, Reuveni et al. (1990) reported that GA_3 or GA_3 and kinetin caused shoot growth to be too slender and long and unsuitable for rooting. They stimulated elongation by including 4.6 μM kinetin and 0.3 μM NAA in 1/2MS medium, and they rooted micropropagated cuttings in the medium supplemented with 4.9 μM BA.

Arora and Singh (1978b) rooted shoots on LS medium devoid of auxin but containing 2.3 μM kinetin and 2.6 μM GA_3. Kinetin was not essential for root initiation, but it stimulated root growth. Yie and Liaw (1977) established roots on shoot cuttings in liquid or agar-gelled MS medium containing 15% CW, 3% sucrose, and 28.5 μM IAA. Roots developed in two weeks. Plants were rinsed of medium and potted in vermiculite. Rajeevan and Pandey (1986) induced roots on micropropagated shoots using IBA. They tested IAA, IBA, NAA, 2-naphthoxyacetic acid (NOA), and 2,4-D in a range of concentrations from about 1.0 to 25 μM, but only 4.9 to 19.7 μM IBA induced roots in 40 to 60% of the cuttings. The plants, however, were stunted.

De Winnaar (1988) obtained roots in one-third of the shoots grown on half-strength Murashige and Tucker medium (Murashige and Tucker 1969) containing 4.9 to 19.7 μM IBA, but only after five subculture passages. Earlier attempts at rooting were not as effective since only 1/10 of the explants rooted with IBA; thus, De Winnaar concluded that rooting improved with time in culture. De Winnaar was not able to acclimate the plants to field conditions due to wilting.

It appears that papayas are most efficiently rooted in MS medium containing 4.9 to 19.7 μM IBA (Litz and Conover 1978a, 1981, Pandey and Rajeevan 1986, De Winnaar 1988, Miller and Drew 1990, Reuveni et al. 1990) or 28.5 μM IAA

(Yie and Liaw 1977) if cuttings are 1- to 2-cm tall (Miller and Drew 1990). Cuttings with leaves rooted readily, especially if they were excised from rooted cuttings in culture (Miller and Drew 1990). In most cases, once roots were established, transfer of plants to the greenhouse and field was easily accomplished.

The literature provides convincing evidence that papayas can be transformed and transgenic plants recovered. The following chapters describe the methods that were used to accomplish these goals.

CHAPTER 3. SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM IMMATURE ZYGOTIC EMBRYOS OF PAPAYA (*CARICA PAPAYA* L.)

3.1 INTRODUCTION

Effective gene transfer systems require reliable and efficient procedures for plant regeneration from transformed cells. Transgenic plants have been regenerated from protoplasts (Toriyama et al. 1988, Zhang and Wu 1988), leaf disks (Horsch et al. 1985, McCormick et al. 1986, Hinchee et al. 1988), and somatic embryos (McGranahan et al. 1988). Prior to these successful transformation attempts, efficient tissue culture procedures for regeneration had been developed for the crops of interest to those groups, including rice, tobacco, tomato, soybean, and walnut.

Transgenic papaya plants might be produced if efficient protocols existed for regeneration from protoplasts, leaf disks, or somatic embryos. Chen et al. (1987) presented data for high frequency regeneration of papaya plants from root callus-derived somatic embryos that produced more than 100 plants per explant. In contrast, other papaya tissues have proven more difficult to regenerate. Protoplasts of papaya leaves have been produced in large numbers, and calli have been recovered at low frequency, but these calli have not regenerated plants (Litz and Conover 1979, Litz 1984). Litz et al. (1983) regenerated plants from shoots obtained

from papaya cotyledon calli but did not report on the efficiency of the procedure. Likewise, no mention of regeneration efficiency is made in reports of plants produced from seedling calli (Arora and Singh 1978a) or from somatic embryos initiated from seedling stem calli (Yie and Liaw 1977). Although Pang and Sanford (1988) produced transgenic papaya calli from leaf disks infected with *Agrobacterium*, they were not able to obtain plants.

Apart from the root cultures reported by Chen et al. (1987), ovule tissues have been the best source of regenerable papaya cultures via somatic embryogenesis. Most papaya "ovular" somatic embryos, reported in the literature to be of nucellar origin (Litz and Conover 1981b, 1982, 1983), have subsequently been shown to derive instead from highly embryogenic zygotes produced in interspecific crosses between papaya and *Carica cauliflora* Jacq. (Moore and Litz 1984, Manshardt and Wenslaff 1989a). However, Litz and Conover (1981b) reported that occasional cultured ovules from self-pollinated papayas also became embryogenic. They did not specify the zygotic or maternal origin of the somatic embryos.

We attempted to improve the efficiency of regeneration from papaya tissue cultures. Low success rates in earlier experiments with leaf disk, cotyledon, and immature ovule culture (unpublished results), and the success of others in inducing embryogenesis in immature zygotes (Tulecke and

McGranahan 1985), led us to try the latter approach with papaya. In this report, we demonstrate the capacity for high frequency somatic embryogenesis in immature embryos of four commercial cultivars of *C. papaya*.

3.2 MATERIALS AND METHODS

3.2.1 PLANT MATERIAL

Immature fruits of *C. papaya* L., 90 to 114 days post-anthesis, were harvested from field-grown trees of four hermaphroditic cultivars, 'Sunrise', 'Sunset', 'Waimanalo', and 'Kapoho'. Hawaiian papaya cultivars are hermaphroditic and highly self-pollinating, although without controlled pollination, a low rate of outcrossing is possible. Age of fruit was estimated by position on the trunk of the tree. Fruits in one experiment were self-fertilized by hand pollination, while in the other experiments, fruits resulted from open pollination.

3.2.2 MEDIA

Media consisted of half-strength and full-strength MS salts (Murashige and Skoog 1962). The half-strength medium also contained 0.3 mM myo-inositol, full strength MS vitamins, 2.7 mM glutamine, 6% sucrose, and 1% Difco

Bactoagar and is referred to as maturation medium. Combinations of supplements to the maturation medium were tested for efficacy in induction of embryogenic growth (Table 3.1). Maturation medium containing 2,4-D is referred to as induction medium. The 2,4-D concentrations used were 0, 0.45, 2.3, 4.5, 22.6, 45.2, 67.9, and 113.1 μM. Twenty percent coconut water (v/v) was added to media in experiments one and two. Additional growth regulators, thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea, Noram Corp, Wilmington, DE), BA (6-benzylaminopurine, Sigma Chemical Co., St. Louis, MO), and picloram (4-amino-3,5,6-trichloropicolinic acid, Dow Chemical Co., Midland, MD), were added to some media in experiment one. Somatic embryo germination medium contained MS salts, 0.6 mM myo-inositol, 3% sucrose, 23.2 μM kinetin (Sigma Chemical Co.), and 1% Difco Bactoagar. Germination medium without kinetin was used for plant enlargement and will be referred to as MS medium. The pH of all media was adjusted to 5.8 prior to autoclaving. About 25 ml of medium were dispensed into sterile 100 x 15-mm petri dishes.

3.2.3 CULTURE CONDITIONS

Immature papaya fruits were soaked in 20% Clorox (1.05% sodium hypochlorite) with two drops of Tween 20 per liter of solution for one hour. Fruits were air-dried in a laminar

flow hood prior to dissection and ovule excision. Excised embryos (Fig. 3.1A) were plated on agar media in replicates of three unless otherwise noted. A single fruit provided all the embryos of a certain age for each experiment. Dishes were sealed with Parafilm and stored in the dark at 27^{o}C. Cultures were scored once a week for six weeks for numbers of embryogenic zygotic embryos.

Four experiments were conducted. The first experiment screened 90- to 114-day-old, open-pollinated embryos from 'Sunrise', 'Sunset', and 'Waimanalo' for production of somatic embryos on induction medium containing 20% coconut water and 0 to 22.6 μM 2,4-D. In addition, different combinations of growth regulators were included in some media. Two to six replicates of plates containing 30 zygotic embryos each were scored to determine the percentage of zygotic embryos that showed embryogenic development. An embryogenic zygotic embryo is defined as one that produced two or more somatic embryos on its apex or radicle.

The second experiment extended the upper range of 2,4-D concentrations to test for frequency of embryogenic zygotic embryos. Embryos from 105-day-old, open-pollinated fruit of 'Sunrise', 'Sunset', 'Waimanalo', and 'Kapoho' were plated on induction medium containing 20% coconut water and 0 to 113.1 μM 2,4-D.

Coconut water was eliminated from the media in the third and fourth experiments that were designed to test the

effect of 2,4-D alone on somatic embryo initiation. Embryos of 'Sunset', 'Sunrise', and 'Waimanalo' were 104-day-old products of self fertilization by hand pollination, while 'Kapoho' embryos were excised from open-pollinated, 105-day-old fruit.

The number of somatic embryos induced per zygotic embryo was recorded after various intervals on induction medium containing 2,4-D. Somatic embryos were transferred to maturation medium for continued development and to germination medium thereafter. Germinated plants enlarged on MS medium under continuous illumination by cool white fluorescent lights, 35 μmol m^{-2} sec^{-1} photosynthetically active radiation (PAR) at 27^{o}C. Shoots with three or more leaves on 2-cm-tall stems were rooted in vermiculite and grown in the greenhouse.

3.3 RESULTS AND DISCUSSION

3.3.1 ZYGOTIC EMBRYO CULTURE

In experiment one, somatic embryos were initiated in all cultures containing 0.45 to 22.6 μM 2,4-D (Fig. 3.1B and 3.1C). Between 49 to 62% of the zygotic embryos from 'Sunrise', 'Sunset', and 'Waimanalo' produced two to 20 somatic embryos on apical domes, in cotyledonary axils, and on root meristems on medium containing 22.6 μM 2,4-D (data

not shown). Other medium supplements, including picloram, BA, thidiazuron, and coconut water, were inhibitory to somatic embryogenesis (Table 3.1). Zygotic embryos plated on phytohormone-free media either remained quiescent or germinated.

Embryos plated on induction medium containing 2.3 μM 2,4-D became swollen at the radicle ends and from this area produced the largest masses of loose brown calli in the treatments. The small number of zygotic embryos that became embryogenic on media containing 2.3 μM 2,4-D with or without coconut water, produced fewer somatic embryos per zygotic embryo compared to cultures on media containing higher 2,4-D concentrations. These somatic embryos frequently were fasciated but were further advanced developmentally than were the somatic embryos initiated with higher 2,4-D treatments.

In the second experiment with coconut-water-containing media, somatic embryos were readily produced in all of the upper ranges of 2,4-D concentrations but at reduced frequencies on media containing more than 22.6 μM 2,4-D. The largest numbers of embryogenic zygotic embryos were produced by each of the four genotypes on medium supplemented with 22.6 μM 2,4-D. The highest percentage of embryogenic zygotic embryos was produced by 'Sunset' with 93% becoming embryogenic (Fig. 3.2). 'Waimanalo' had the

lowest yield of 57%. Higher 2,4-D concentrations were apparently inhibitory to embryogenesis.

Cultures grown on media not supplemented with coconut water produced embryogenic zygotic embryos that were similar in appearance to those observed on coconut water-containing media. Embryogenesis occurred in the same range of 2,4-D concentrations, 2.3 to 113.1 μM, as in media containing coconut water, but the optimum auxin concentration varied from 4.5 μM for 'Sunset', 'Waimanalo', and 'Kapoho' to 22.6 μM for 'Sunrise' (Fig. 3.3). The overall response of 'Kapoho' was considerably lower compared to results from experiment two (Fig. 3.2).

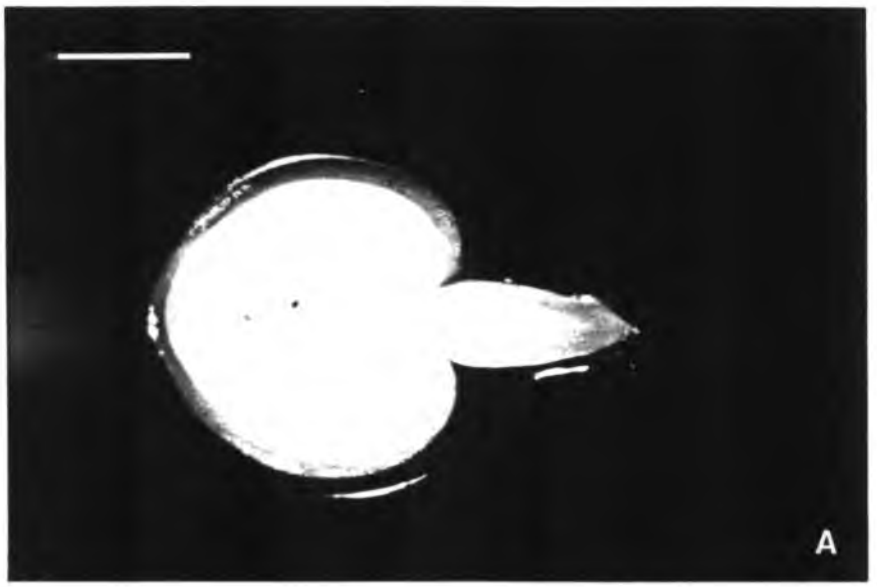
In a fourth experiment, about 50% of the zygotic embryos of 'Kapoho' selections 'KS158', 'HCAR27', and 'KS' produced somatic embryos on media containing 22.6 μM 2,4-D, and this percentage increased to 80 to 100% of the embryos plated on 45.2 to 113.1 μM 2,4-D (Fig. 3.4). Contrary to the results of the previous experiments, the higher 2,4-D concentrations were not inhibitory to somatic embryo formation. It is not known why the differences in optimum auxin concentration between experiments occurred, but seasonal variation in zygote developmental stage could affect somatic embryogenesis. The method used for estimating embryo age is subject to seasonal variation.

Table 3.1. Percentage of immature zygotic embryos that became embryogenic after five to six weeks of culture on maturation medium supplemented with phytohormones and/or coconut water

		Media supplements[a]					
CV	Med.	CW	BA	TD	2,4-D	PIC	Emb. ZE (%)
SR	1	-	-	-	+	-	40
	2	-	+	-	+	-	5
	3	+	+	-	+	-	5
	4	+	-	-	+	-	20
	5	-	-	+	+	-	2
	6	-	+	-	-	+	2
	7	+	-	-	-	+	0
	8	-	-	+	-	+	10
SS	1	-	-	-	+	-	37
	2	-	+	-	+	-	2
	3	+	+	-	+	-	1
	4	+	-	-	+	-	28
	5	-	-	+	+	-	28
	6	-	+	-	-	+	0
	7	+	-	-	-	+	6
	8	-	-	+	-	+	11
W	1	-	-	-	+	-	47
	2	-	+	-	+	-	0
	3	+	+	-	+	-	0
	4	+	-	-	+	-	25
	5	-	-	+	+	-	0
	6	-	+	-	-	+	4
	7	+	-	-	-	+	3
	8	-	-	+	-	+	1

[a]Supplements: CW = 20% coconut water, BA = 17.8 μM 6-benzylaminopurine, TD = 0.09 μM thidiazuron, 2,4-D = 4.5 μM 2,4-dichlorophenoxyacetic acid, PIC = 2.1 μM picloram
CV = cultivar, Med. = medium, Emb. ZE (%) = percentage of embryogenic zygotic embryos, SR = 'Sunrise', SS = 'Sunset', W = 'Waimanalo'
The pluses and minuses represent presence or absence of media supplements

Fig. 3.1. Papaya regeneration via 2,4-D-induced somatic embryogenesis in immature zygotic embryos. Induction medium contained 22.6 μM 2,4-D and 20% coconut water. Scale = 1 mm. A) Immature 'Waimanalo' zygotic embryo excised from ovule 75 days after anthesis and photographed after three days of culture on maturation medium. B) Enlarged, embryogenic apical dome of 'Sunset' zygotic embryo excised 114 days after anthesis and photographed after 17 days on induction medium.


A

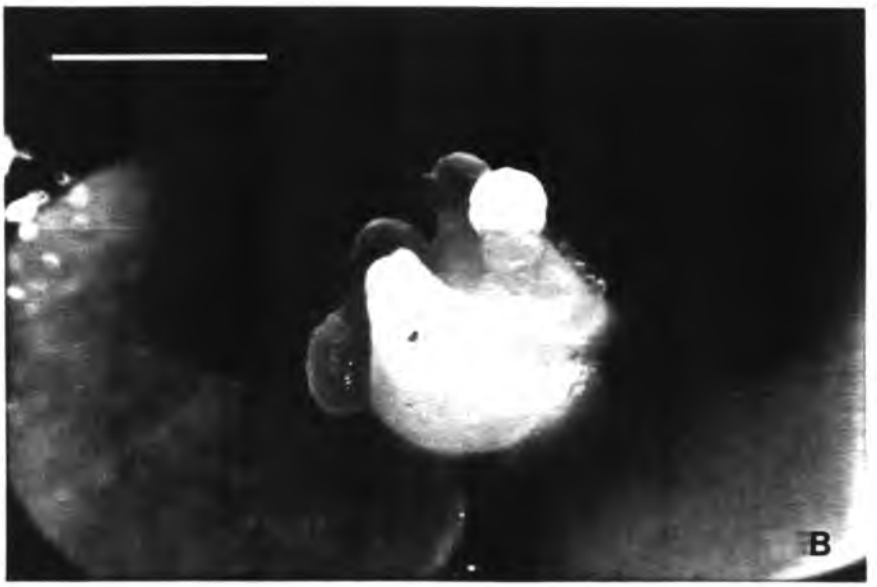

B

Fig. 3.1. (Continued) Papaya regeneration via 2,4-D-induced somatic embryogenesis in immature zygotic embryos. Induction medium contained 22.6 μM 2,4-D and 20% coconut water. Scale = 1 mm. C) Somatic embryos budding from apical dome of 'Sunrise' zygotic embryo excised 93 days after anthesis and photographed after five weeks of culture on induction medium. D) Large number of somatic embryos produced from one 'Sunset' zygotic embryo after five months of culture on induction medium.


C

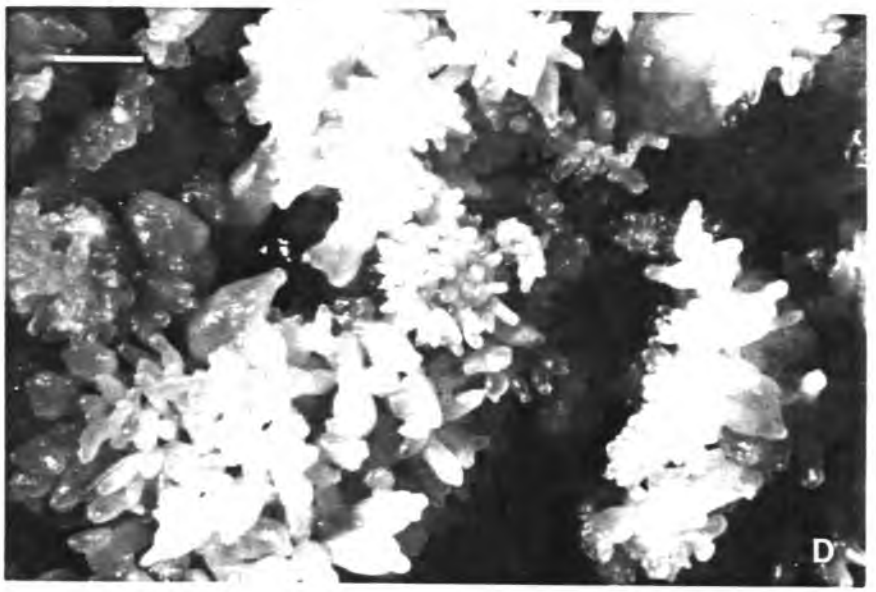

D

Fig. 3.1. (Continued) Papaya regeneration via 2,4-D-induced somatic embryogenesis in immature zygotic embryos. Induction medium contained 22.6 μM 2,4-D and 20% coconut water. Scale = 1 cm. E) Papaya shoot produced from a somatic embryo derived from the apical dome of a 'Kapoho' zygotic embryo cultured on induction medium.


E

Fig 3.2. Percentage of zygotic embryos that developed embryogenic growth in four papaya cultivars after five to six weeks of culture on induction medium containing 20% coconut water and 0 to 113.1 μM 2,4-D. Error bars represent $\pm$ one standard error.

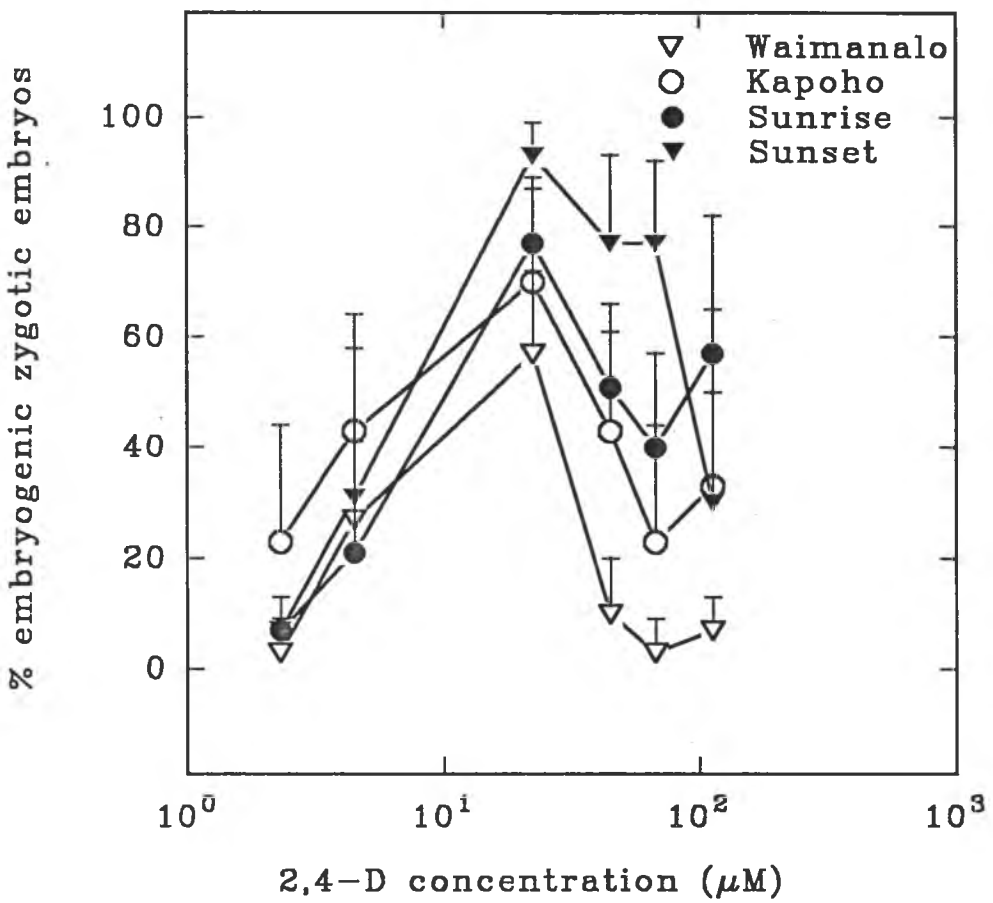

% embryogenic zygotic embryos
100
80
60
40
20
0
10^0
10^1
10^2
10^3
2,4–D concentration (μM)
Waimanalo
Kapoho
Sunrise
Sunset

Fig. 3.3. Percentage of zygotic embryos that developed embryogenic growth in four papaya cultivars after five to six weeks of culture on induction medium containing 0 to 113.1 μM 2,4-D. Error bars represent $\pm$ one standard error.

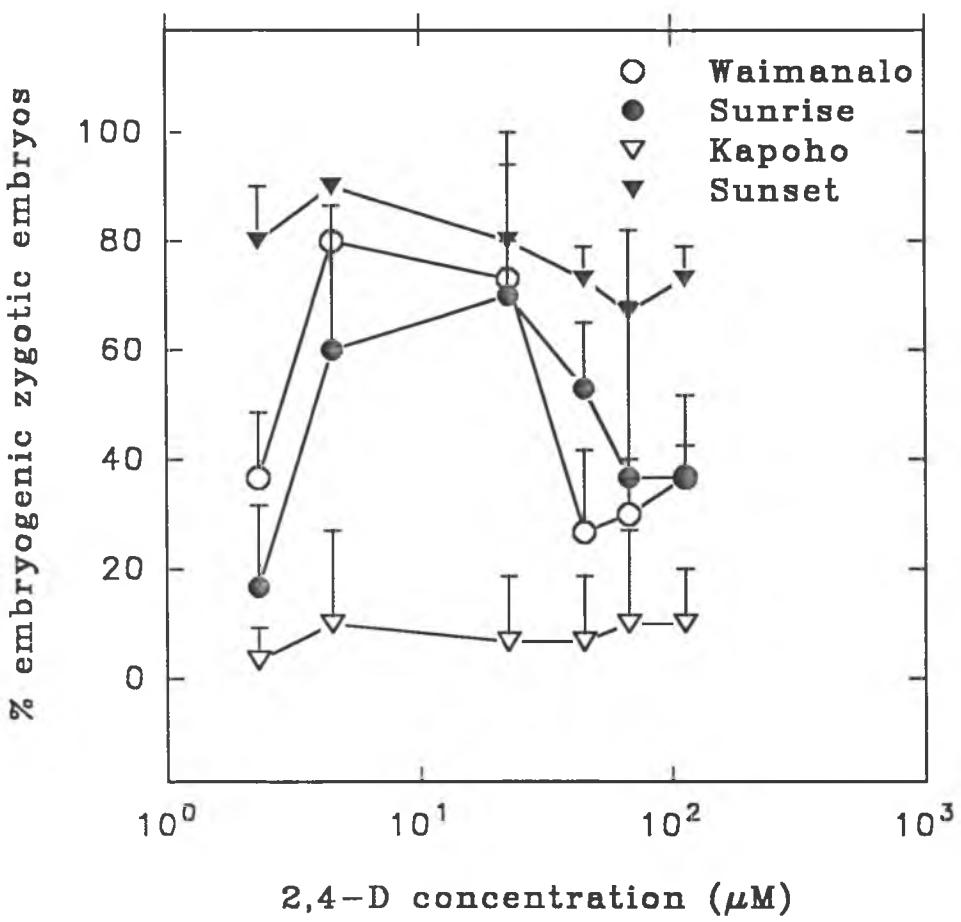
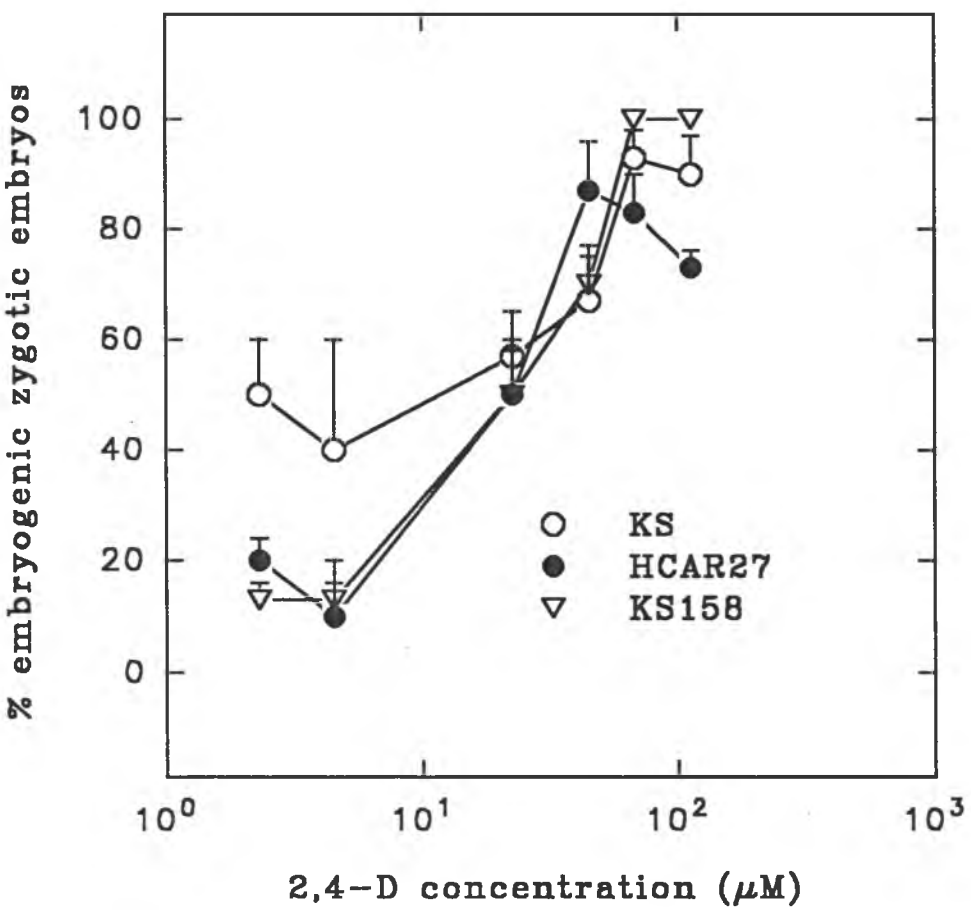

Waimanalo
Sunrise
Kapoho
Sunset
% embryogenic zygotic embryos
100
80
60
40
20
0
10^0
10^1
10^2
10^3
2,4-D concentration (μM)

Fig. 3.4. Percentage of zygotic embryos that developed embryogenic growth in three selections of the cultivar 'Kapoho' after six weeks of culture on induction medium containing 0 to 113.1 μM 2,4-D. Error bars represent $\pm$ one standard error.


% embryogenic zygotic embryos
100
80
60
40
20
0
10^0
10^1
10^2
10^3
2,4-D concentration (μM)
KS
HCAR27
KS158

3.3.2 SOMATIC EMBRYO CULTURE

Numbers of somatic embryos produced per zygotic embryo were not recorded; however, three weeks after culture initiation in all experiments, two to 20 globular embryos were observed (Fig. 3.1B). Similarly, at the end of the experiments, six-week-old cultures showed zygotic embryos bearing up to 50 embryos. In five months, 200 to 400 embryos could be counted on each of five zygotic embryos on a single randomly selected plate containing 67.9 μM 2,4-D. Figure 3.1D shows a portion of the somatic embryos that resulted from a single zygotic embryo of 'Sunset' cultured for four months on induction medium containing coconut water and 67.9 μM 2,4-D and photographed one month after subculturing to fresh medium.

Somatic embryos transferred to phytohormone-free maturation medium developed and enlarged. Cotyledons enlarged to 2 to 3 mm in diameter, but somatic embryos remained ivory-colored, and germination rarely occurred. After subsequent transfer to germination medium, radicles and apices of the somatic embryos became active in one to four weeks, cotyledons turned dark green, and shoots elongated. Rooting occurred, but frequently the roots were loose-celled and large. More often, watery, large-celled calli rather than roots developed at the radicle ends of somatic embryos. Nevertheless, germinated embryos developed

into normal-appearing papaya shoots on MS medium when grown under continuous light (Fig. 3.1E). Shoots of the four cultivars rooted in one to three weeks in vermiculite and were grown in the greenhouse.

High frequency somatic embryogenesis has been achieved previously in papaya tissue cultures derived from immature zygotic embryos and seedling explants. Chen et al. (1987) observed root-derived somatic embryos after three months of culture on NAA- and BA-containing MS medium. Litz and Conover (1982, 1983) obtained somatic embryos from *C. papaya* x *C. cauliflora* hybrid ovules cultured for three months on half-strength MS or White's medium supplemented with 20% coconut water. De Bruijne et al. (1974) and Yie and Liaw (1977) did not mention the length of time required for producing their somatic embryos. In our experiments, embryogenesis was observed after only three weeks of culture; thus it occurred more rapidly than in earlier reports. Rapid embryogenesis with our protocol is attributed to two factors: the removal of immature zygotic embryos from maternal ovular tissues and the inclusion of 2,4-D in the culture medium. Excision of zygotic embryos from maternal ovular tissues resulted in quicker development of somatic embryos than reported for *C. papaya* x *C. cauliflora* hybrids. This may due to closer contact between the exposed, inducible tissues and medium components effecting embryogenesis, particularly 2,4-D.

Our results are consistent with the report by Ammirato (1983) that immature embryos of many plant species can be induced to undergo somatic embryogenesis. Furthermore, Ammirato pointed out that 2,4-D has a significant role in the initiation of somatic embryos from immature zygotic embryos. The widespread success in using immature embryos to produce somatic embryos is also documented by Williams and Maheswaran (1986) who listed about 20 dicotyledonous species in which somatic embryos have been produced from various tissues of immature embryos. We believe our report is the first to document the high potential for somatic embryogenesis exhibited by the apical dome and surrounding tissues of an immature zygotic embryo. Hypocotyls (Freyssinet and Freyssinet 1988) and cotyledons (Tulecke and McGranahan 1985, Finer 1987) frequently are the sources of somatic embryos in immature embryo cultures reported to date.

The papaya regeneration system that we report can be integrated into a useful gene transfer technology. Hawaiian papaya cultivars are highly inbred, and consequently, seedlings faithfully reproduce the parental genotype. This fact makes zygotic embryos a suitable target tissue for practical genetic transformation using using *Agrobacterium* as reported by McGranahan et al. (1988) or other DNA transfer systems. Embryogenic apical domes, for example,

are potential targets for particle gun-mediated transformation (Sanford 1988).

CHAPTER 4. HIGH FREQUENCY SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM PAPAYA HYPOCOTYL CALLUS

4.1 INTRODUCTION

Success in gene transfer technology has been dependent, in part, on efficient plant regeneration from tissue cultures. Pang and Sanford (1988) showed that papaya leaf disks could be transformed by *Agrobacterium*. Following co-cultivation, they detected nopaline in kanamycin-resistant calli, but the calli proved unsuitable for regeneration of plants. One approach that can result in high frequency recovery of plants from cultured tissues is induction of somatic embryogenesis. Williams and Maheswaran (1986) reported that species in at least 20 dicotyledonous families have been induced to develop somatic embryos in tissue culture. Often, the key component for the induction of somatic embryogenesis has been the inclusion of the growth regulator 2,4-D in induction media (Ammirato 1983).

Infection of somatic embryos with *Agrobacterium tumefaciens* could be a useful technique for effecting transformation in dicots, as McGranahan et al. (1988) have demonstrated with walnuts. The *Agrobacterium*-infected walnut cultures were induced to undergo repetitive cycles of somatic embryogenesis to enhance the recovery of wholly transformed, non-chimeric plants. It is likely that

transformation of a more embryogenic callus would permit regeneration of transgenic papaya plants.

Transgenic maize (Gordon-Kamm et al. 1990, Fromm et al. 1990), sunflower (Finer and McMullen 1990), and papaya (Chapter 5) have been reported following particle bombardment of somatic embryos or embryogenic calli. The somatic embryo cultures that were bombarded in the latter work were developed from immature zygotic embryos (Chapter 3) and from hypocotyl sections cultured on media containing 2,4-D (this report).

High frequency somatic embryogenesis and plant regeneration in papaya have been described for root cultures (Chen et al. 1987) and immature zygotic embryos (Chapter 3). De Bruijne et al. (1974), Yamamoto and Tabata (1989), Yie and Liaw (1977), and Mehdi and Hogan (1979) also produced papaya somatic embryos. The latter two groups reported plant regeneration; however, frequencies of induction were not reported. As an adjunct to the zygotic embryo work, high frequency somatic embryogenesis is now reported in papaya callus cultures produced from hypocotyl sections of ten-day-old seedlings. The Hawaiian papaya cultivars 'Kapoho', Sunrise', 'Sunset', and 'Waimanalo' produced embryogenic hypocotyl calli, but the optimal response of each to 2,4-D and sucrose concentrations differed.

4.2 MATERIALS AND METHODS

4.2.1 PLANT MATERIAL AND CULTURE CONDITIONS

Seeds from self-pollinated Hawaiian papaya cultivars, 'Kapoho', 'Sunrise', 'Sunset', and 'Waimanalo', were surface-sterilized in batches of 50 in 50 ml of 20% Clorox (1.05% sodium hypochlorite) containing two drops of Tween 20 per liter of solution. They were shaken (100 rpm) for one hour at 27°C, and seeds with dark, unbleached spots or holes were discarded. The remaining seeds were shaken for 24 hours at 27°C in 50 ml of sterile 1.0 M KNO_3 (Nagao and Furutani 1986). Floating seeds were discarded, and submerged seeds were shaken in 100 ml of sterile water at 32.2°C for three days or until the testae cracked (Nagao and Furutani 1986). The imbibed seeds were germinated, five per 50 ml of 1% water agar in 150-ml specimen jars, under cool white fluorescent lights, photosynthetically active radiation (PAR) = 35 μmol m^{-2} s^{-1} at 27°C. The imbibition water was streaked on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) to identify contaminated seed batches.

In about ten days, seedlings with expanded cotyledons and one 2- to 4-mm-long trilobed leaf (Fig. 4.1A) were dissected for explanting. The 3- to 12-cm-long hypocotyls were sectioned into 2- to 3- or 4- to 5-mm lengths, and the

sections were plated on various media listed in Table 4.1. All of the sections from one seedling were plated in one 100 x 15-mm petri dish containing about 25 ml of medium (Fig. 4.1B). Between 15 and 60 2-mm sections were plated, depending on the length of the hypocotyl.

Cotyledons and shoot apices were frequently plated with the hypocotyl sections to determine the response of these tissues to culture. Cotyledons were either plated whole or sliced into 2- to 3-mm-wide sections. Shoot apices were about 4 mm in length and included the cotyledonary axils. Roots were severed from the hypocotyls and plated in separate culture dishes.

4.2.2 MEDIA

Seeds were germinated on 1% Difco Bactoagar, later changed to 0.5% Sigma A1296 agar due to unavailability of the former medium. Callus induction media consisted of half-strength and full-strength MS salts (Murashige and Skoog 1962). Half-strength MS salts medium supplemented with 0.3 mM myo-inositol, 26.7 μM glycine, 4.1 μM nicotinic acid, 2.4 μM pyridoxine·HCl, 1.2 μM thiamine·HCl, and 1% Difco Bactoagar (later 0.5% Sigma A1296 agar), pH 5.8, is referred to as 1/2MS organics medium. When supplemented further with 2.7 mM glutamine and 6% sucrose, it is called maturation medium. Concentrations of 2,4-D tested for

embryogenic callus induction were 0, 2.3, 4.5, 22.6, 45.2, 67.9, and 113.1 μM. Maturation media containing 2,4-D and various concentrations of sucrose are referred to as induction media. Other growth regulators were tested in combination with each other and with 2,4-D. A list of concentrations and combinations of growth regulators tested is given in Table 4.1.

Sucrose concentration was 3, 6, 7, 8, or 9%, while the 2,4-D concentration was kept constant at 4.5 μM in one experiment with 'Kapoho'. The osmotic potentials of the respective media were 0.1, 0.2, 0.23, 0.27, and 0.3 MPa, respectively, as determined with a Wescor model 5100B vapor pressure osmometer. In other experiments, both 2,4-D and sucrose concentrations were varied in factorial designs with 0 to 113.1 μM 2,4-D and 3, 6, or 9% sucrose in induction media.

Embryogenic calli were sequentially transferred to maturation and MS media. MS medium consisted of MS salts, 0.6 mM myo-inositol, 1.2 μM thiamine·HCl, 3% sucrose, and 0.5% Sigma A1296 agar, pH 5.8. Shoot cuttings were micropropagated on MS medium or in liquid micropropagation medium (MPII). The MPII medium consisted of MS salts, 0.6 mM myo-inositol, 3.0 μM thiamine·HCl, 0.9 μM BA, 0.5 μM kinetin, and 2% sucrose, pH 5.8 (Lee 1987). Cuttings were callused and rooted in MS medium supplemented with 4.9 μM IBA. Germinated somatic embryos and rooted cuttings were

Table 4.1. Concentration ranges of supplements in half-strength MS media tested for induction of somatic embryogenesis in papaya seedling cultures

AUXIN (μM)	CYTOKININ (μM)	%SUCROSE	No. of CULTURES
NAA 0.13-10.7 ± 2,4-D 4.5	BA 0.9-2.3[a] or TD 0.009-0.9 or 2iP 9.8-49.2	1.5-3	64
PIC 0.008-2.1	TD 0.09-0.9 or BA 0.9-17.8 or CW 20% or no cytokinin	1.5-6	140
no auxin	BA 0.9-1.1 or K 4.6 or TD 0.09 or no cytokinin	1.5-9	66
2,4-D 0.5-4.5	BA 0.9-1.1 or CW 20% or TD 0.023-0.9	1.5-6	66
2,4-D 2.3-113	no cytokinin	3-9	351
NAA 0.05 or 2,4-D 4.5 or no auxin	BA 0.4 or no cytokinin	3-6	25

[a]Cefotaxime included in some media, 0.4 mM
NAA = 1-napthaleneacetic acid
PIC = picloram = 4-amino-3,5,6-trichloropicolinic acid
2,4-D = 2,4-dichlorophenoxyacetic acid
BA = 6-benzylaminopurine
TD = thidiazuron = N-phenyl-N'-1,2,3-thidiazol-5-ylurea
2iP = 6-(gamma,gamma-dimethylallyloamino)purine
K = kinetin
CW = coconut water

grown in sterile commercial vermiculite moistened with MS liquid medium (MSV). The vermiculite medium was prepared by autoclaving equal volumes of dry vermiculite and MS liquid medium.

4.2.3 SOMATIC EMBRYOGENESIS, PLANT REGENERATION, AND MICROPROPAGATION

Plates of hypocotyl sections were incubated in the dark at 27°C. Each plate contained all of the hypocotyl sections from a single seedling, and one plate constituted one sample replicate. Embryogenic development was assessed eight to 14 weeks after culture initiation.

Data were calculated in two ways for each concentration of 2,4-D. The first set of data consisted of percentage of hypocotyl sections showing embryogenic development on each plate. Percentages were averaged for all sample plates of a particular cultivar at a particular 2,4-D concentration and the means (with standard errors) were plotted against 2,4-D concentrations. The second method of analyzing the hypocotyl data involved calculating the percentage of plates that contained at least one embryogenic hypocotyl section. This was necessary in order to compare the hypocotyl data with other kinds of explants, which were scored as positive for embryogenesis if they contained at least one embryogenic callus. The data for each cultivar were summarized as the

percentage of plates that showed embryogenic development at each 2,4-D concentration. In plates containing hypocotyls as well as other explants, the responses of the hypocotyls were recorded independently of the other explants.

A mixed sample of seedlings from two papaya cultivars, 'Kapoho' and 'Sunrise', was explanted as 4- and 2-mm hypocotyl sections, and the data were compared to determine if explant length affected frequency of embryogenesis. The students' t test was applied to determine significance of the difference between means at each 2,4-D concentration.

Somatic embryos arising from embryogenic calli developed on maturation medium and germinated on MS medium. Shoots from regenerated plants were micropropagated in two ways. One-cm-tall shoots were placed in liquid micropropagation medium and suspended at 100 rpm. Profuse lateral branches, 1- to 2-cm long, were removed to MS medium containing 4.9 μM IBA for root initiation. The rooted cuttings were grown in MSV. Before tranfer to the greenhouse, the rooted plants were rinsed of media, potted in commercial potting mix, and enclosed in plastic bags for about a week. When new leaves emerged, the corners of the bags were cut and the holes were gradually enlarged to acclimate the plants to ambient humidity. Plants were grown in the greenhouse and transplanted to the field at the University experiment station at Poamoho, Hawaii.

4.3 RESULTS AND DISCUSSION

4.3.1 CALLUS INDUCTION

All media, including those without growth regulators, supported callus development on hypocotyl sections of the four Hawaiian cultivars, 'Kapoho', 'Sunrise', 'Sunset', and 'Waimanalo'. Calli developed initially from the cut surfaces of the explants, but the appearance of the calli differed, depending on the growth regulator treatment. Calli that developed on induction media and on picloram-containing media differed in color and texture from calli induced on other media. In the first two to three weeks of culture, the calli were wet-looking, light brown or yellow in color and sometimes resembled glistening bacterial colonies, whereas calli on growth regulator-free medium were white and dry in appearance, with cells that resembled a loose tangle of epidermal hairs when observed with the aid of a microscope. The calli that formed without growth regulators appeared to stop growing after about three weeks. In addition to limited callusing on cut surfaces, many of the hypocotyl sections cultured without growth regulators developed adventitious roots close to the basal end (Fig. 4.1C); explants cultured on the 9% sucrose-containing medium rarely developed roots. Growth regulators 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BA),

kinetin (K), 6-(gamma,gamma-dimethylallylamino)purine (2iP), thidiazuron (TD), and combinations of these compounds induced white, crystal-like calli.

Several auxin-like growth regulators were tested for ability to induce embryogenic callus in hypocotyl sections. Picloram was used at concentrations of 0.008, 0.08, 0.4, and 0.8 μM in 1/2MS organics media containing 6% sucrose. Roots and wet, brown or crystalline white calli developed, but no embryogenesis occurred after more than five months. Hypocotyl sections of all four papaya cultivars produced roots when plated on media containing 3% sucrose and 0, 0.008, and 0.08 μM picloram. The highest concentration of picloram, 0.8 μM, inhibited root formation. In this experiment, each treatment was applied to hypocotyl sections prepared from two to four seedlings.

Although picloram did not induce embryogenic calli in hypocotyl sections, three out of 20 cotyledon cultures produced one somatic embryo apiece after three months on 1/2MS organics medium containing 1.5% to 3% sucrose, 0.008 to 0.08 μM picloram, 2.7 mM or 6.8 μM glutamine, and 0.9 μM BA. Therefore, picloram may be suitable for embryogenic callus induction in hypocotyl sections after varying sucrose and/or picloram concentrations.

In addition, although NAA was not effective in inducing embryogenesis in this investigation, adjustments of the auxin and/or osmoticum concentrations in the medium may

result in embryogenesis, since De Bruijne et al. (1974), Yie and Liaw (1977), Mehdi and Hogan (1979), Litz et al. (1983), and Chen et al. (1987) have reported embryogenesis and organogenesis in papaya cultures induced with this auxin.

4.3.2 EMBRYOGENIC CALLI FROM HYPOCOTYL SECTIONS

Explant length, and sucrose and 2,4-D concentrations, affected the amount of embryogenic calli produced in hypocotyl sections. The presence of 2,4-D in induction media was necessary for the development of embryogenic hypocotyl calli. Embryogenic calli were observed four weeks after culture initiation, but most of the data were recorded after eight weeks of culture, since the number of hypocotyl sections developing embryogenic callus increased with time (Fig. 4.2 and Fig. 4.1D and 4.1E). Embryogenic calli produced somatic embryos directly on induction media, or they developed somatic embryos after subculture to maturation medium (Fig. 4.1F).

'Kapoho', 'Sunrise', and 'Sunset' produced embryogenic hypocotyl calli on induction media supplemented with up to 22.6 μM 2,4-D (Fig. 4.3). Kapoho' hypocotyl sections formed embryogenic callus with increasing 2,4-D up to 113.1 μM, a concentration at which nearly 100% of the hypocotyl sections produced embryogenic callus. There was no apparent inhibition of embryogenesis in 'Kapoho' at high 2,4-D

concentrations. Up to 76% of 'Sunset' hypocotyl sections produced embryogenic calli in cultures containing 45.2 μM 2,4-D; however, at higher concentrations of the growth regulator, the embryogenic response declined (Fig. 4.3). 'Sunrise' produced lower percentages of embryogenic calli than did 'Kapoho' or 'Sunset'. A maximum of 45% of its hypocotyl sections became embryogenic at 4.5 μM 2,4-D. Higher concentrations appeared to be increasingly inhibitory to embryogenesis (Fig 4.3). Concentrations of 2,4-D above 45.2 μM appear to be phytotoxic to 'Sunrise' and 'Sunset' but not to 'Kapoho'.

'Waimanalo' was not tested as extensively as the other three cultivars. A few 'Waimanalo' hypocotyl sections became embryogenic. In an experiment using 3% sucrose and 2.3 μM 2,4-D in induction media, 8.5 ± 5.6% of the hypocotyl sections became embryogenic, while embryogenesis was not observed in cultures on media containing 3, 6, or 9% sucrose and 45.2 μM or 113.1 μM 2,4-D (Table 4.2). In addition, 6.7 ± 1.9% of the 'Waimanalo' hypocotyl sections became embryogenic on induction medium containing 4.5 μM 2,4-D, 1.8 μM BA, 6% sucrose, and 20% coconut water. It is likely that 'Waimanalo' can be induced to develop higher frequencies of embryogenesis with adjustments of the sucrose and 2,4-D concentrations.

Increasing sucrose concentration stimulated embryogenesis up to optimal levels specific for each

genotype (Figs. 4.3 and 4.4). When the 2,4-D concentration was 4.5 μM, an increase in sucrose concentration produced an increase in the percentage of embryogenic 'Kapoho' hypocotyl sections up to a maximum of 69% in the 7% sucrose treatment (Fig. 4.4). Hypocotyls cultured in medium containing 3% sucrose produced a watery, nonembryogenic callus (Fig. 4.4). These data are in agreement with the reports by Litz (1986a), Close and Ludeman (1987), Close et al. (1989), and Finer (1987) who observed increased embryogenesis with increases in osmotic potential of the culture media. Levels of the phytohormone abscisic acid (ABA) rise with increase in osmotic potential, and that response is hypothesized to stimulate embryogenesis (Ammirato 1983, Close et al. 1989).

In general, explants 2-mm long became embryogenic more frequently than explants 4-mm long (Fig. 4.5). The data for explant length were complicated by the unintentional randomization of cultivars in the 4-mm-section treatment. The percentages of a mixture of 2-mm sections of 'Kapoho' and 'Sunrise' that became embryogenic on media containing 1.8 to 4.5 μM 2,4-D were higher than the percentages of 4-mm sections of the randomized sample of two cultivars (Fig. 4.5). There was a significant difference between means at the 5% level for the concentrations 1.8 μM to 4.5 μM and no embryogenic calli formed on media containing no 2,4-D. Therefore, the 2-mm length was used in all subsequent experiments.

Fig. 4.1. Papaya regeneration via 2,4-D-induced somatic embryogenesis in hypocotyl-derived callus. Scale = 1 cm. A) Ten-day-old papaya seedlings germinated on 1% water agar. Hypocotyls and other tissues were explanted from seedlings at this stage of development. B) Freshly explanted hypocotyl sections, 2-mm long, on induction medium.


A

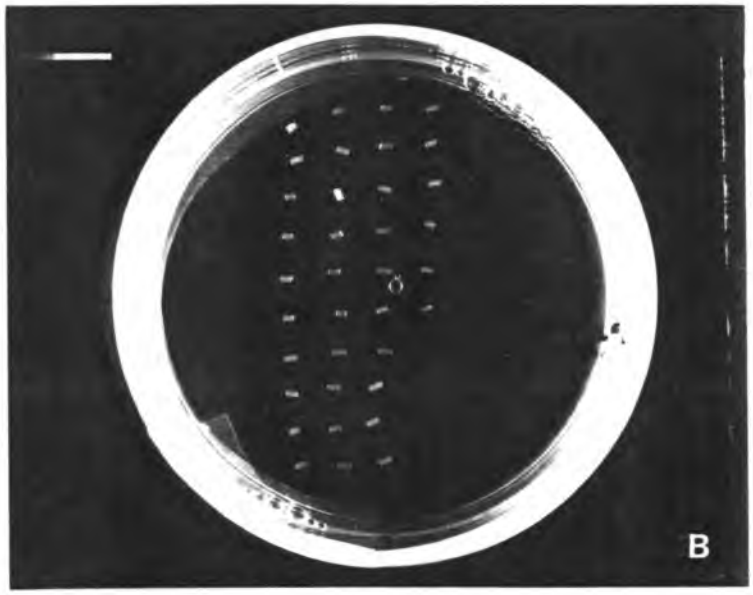

B

Fig. 4.1. (Continued) Papaya regeneration via 2,4-D-induced somatic embryogenesis in hypocotyl-derived callus. Scale = 1 cm. C) Hypocotyl sections on growth regulator-free medium two months after explanting. Hypocotyl sections developed white, fluffy calli on cut surfaces and a few have rooted near the basal end. Sections were alligned with basal ends to the left.

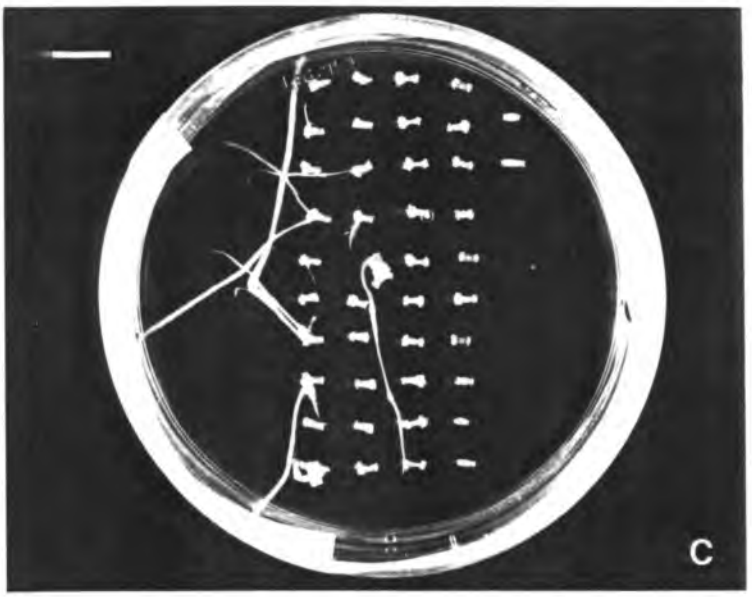
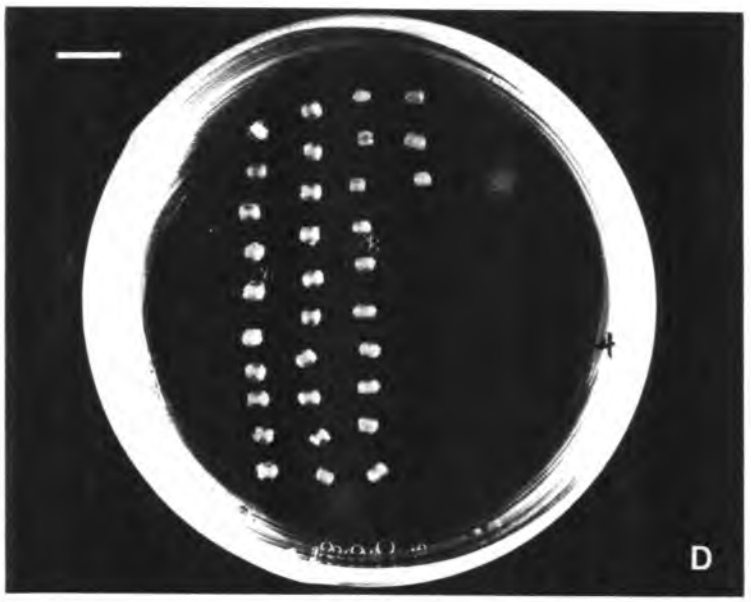

C

Fig. 4.1. (Continued) Papaya regeneration via 2,4-D-induced somatic embryogenesis in hypocotyl-derived callus. Scale = 1 cm. D) Two-week-old culture of 'Kapoho' on induction medium containing 4.5 μM 2,4-D. Hypocotyl sections were uniformly swollen and appeared wet, but no embryogenic calli were visible. E) Same culture as in Fig 4.1D, photographed after two months of culture. Each hypocotyl section developed profuse amounts of embryogenic calli from both cut surfaces.


D

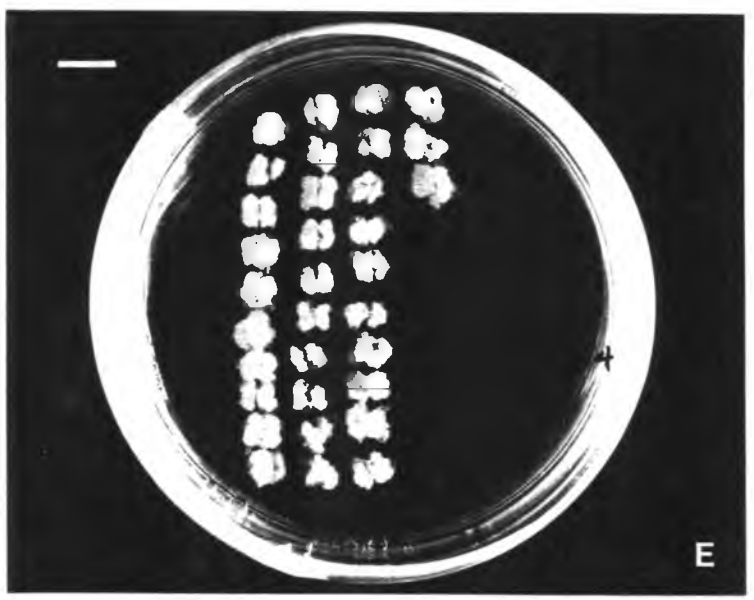

E

Fig. 4.1. (Continued) Papaya regeneration via 2,4-D-induced somatic embryogenesis in hypocotyl-derived callus. Scale = 1 mm. F) Somatic embryos of 'Kapoho' that developed directly on induction medium containing 4.5 μM 2,4-D two months after culture initiation.

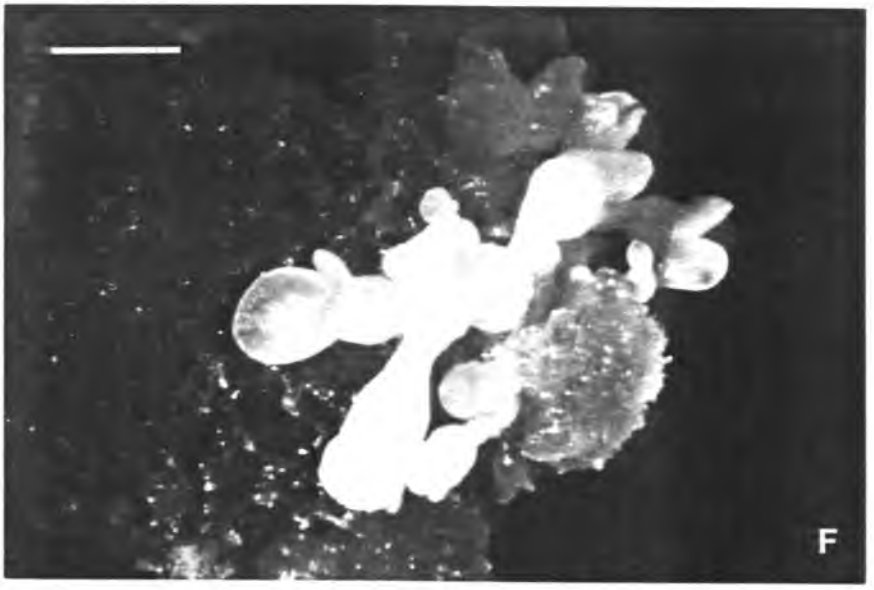

F

Figure 4.2. Effect of 2,4-D on induction of somatic embryogenesis in 'Kapoho' hypocotyl sections. Each dataset represents one culture that contains hypocotyl sections from a single papaya seedling.

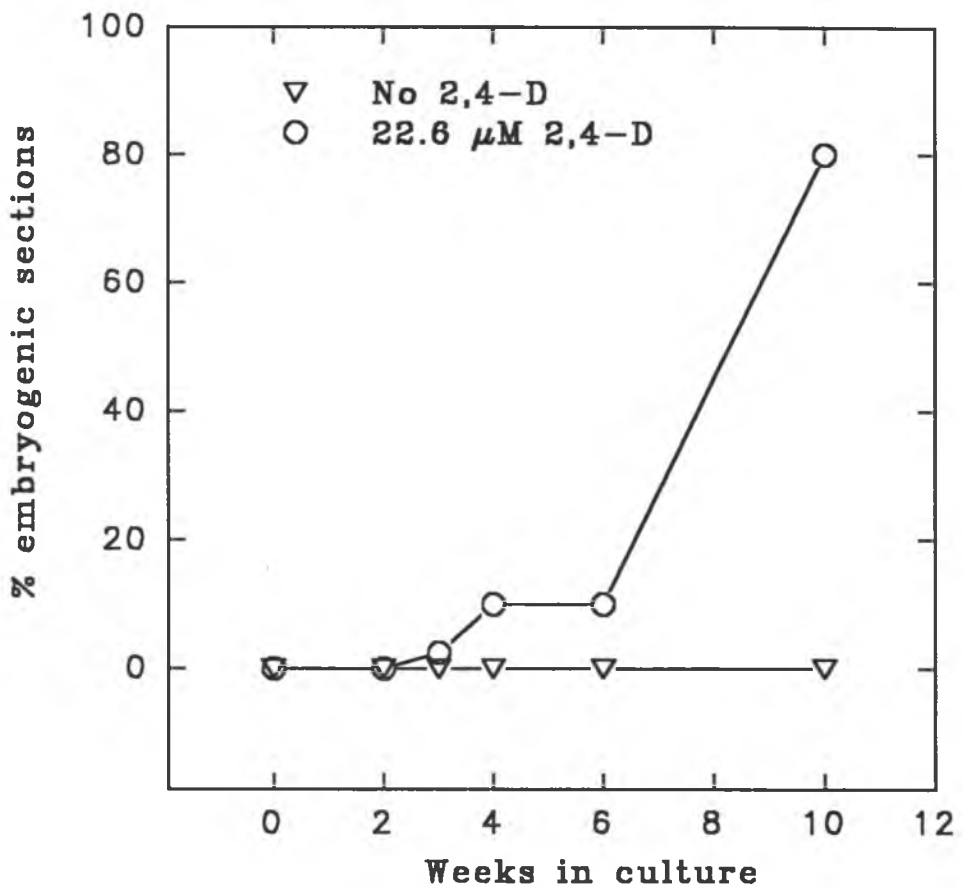

100
80
60
40
20
0
% embryogenic sections
No 2,4-D
22.6 μM 2,4-D
0
2
4
6
8
10
12
Weeks in culture

Fig. 4.3. Effect of 2,4-D concentration on the percentage of hypocotyl sections of three Hawaiian hermaphrodite cultivars induced to embryogenesis after two and a half to three months of culture on induction medium containing 6% sucrose. Means and standard errors were calculated from five to seven samples per treatment for 'Kapoho' and from four to 13 samples for both 'Sunrise' and 'Sunset', except for 'Sunset' at 113.1 μM 2,4-D, for which only two samples were averaged. Bars represent one standard error.

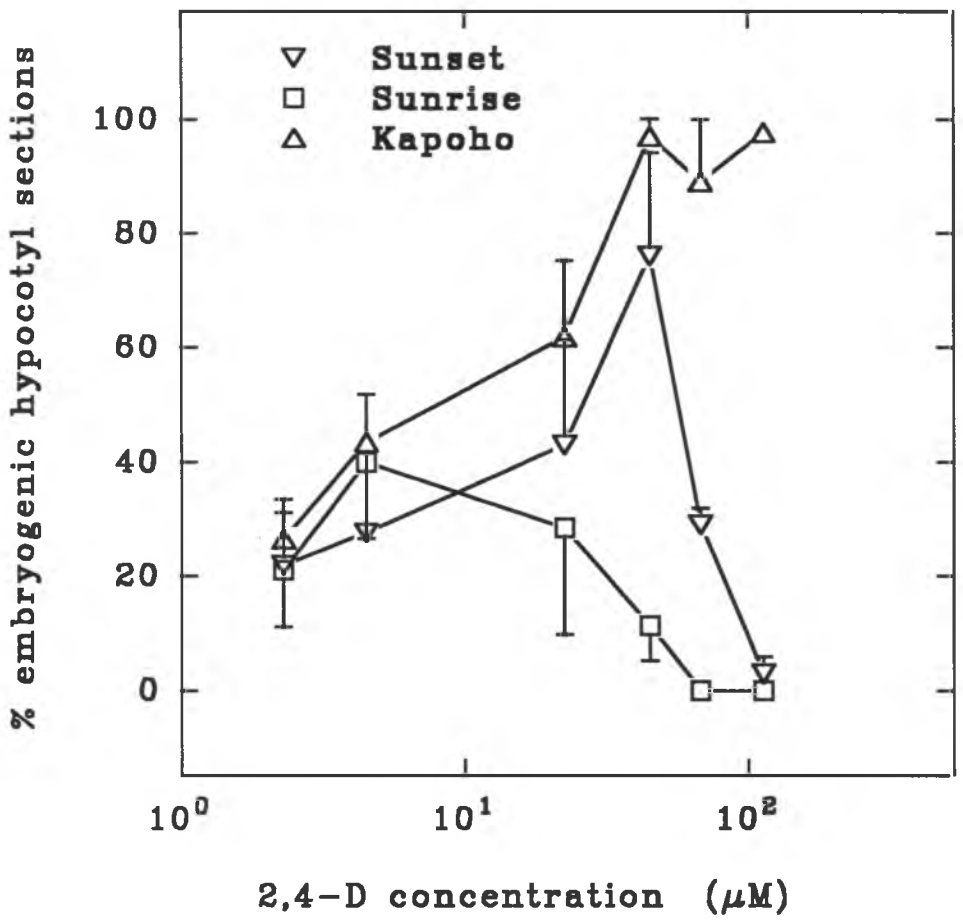

Sunset
Sunrise
Kapoho
% embryogenic hypocotyl sections
100
80
60
40
20
0
10^0
10^1
10^2
2,4-D concentration (μM)

Fig. 4.4. Effect of sucrose concentration on induction of somatic embryogenesis in 'Kapoho' hypocotyl sections after two months of culture on induction media containing 4.5 μM 2,4-D. Data are means of three replicates.

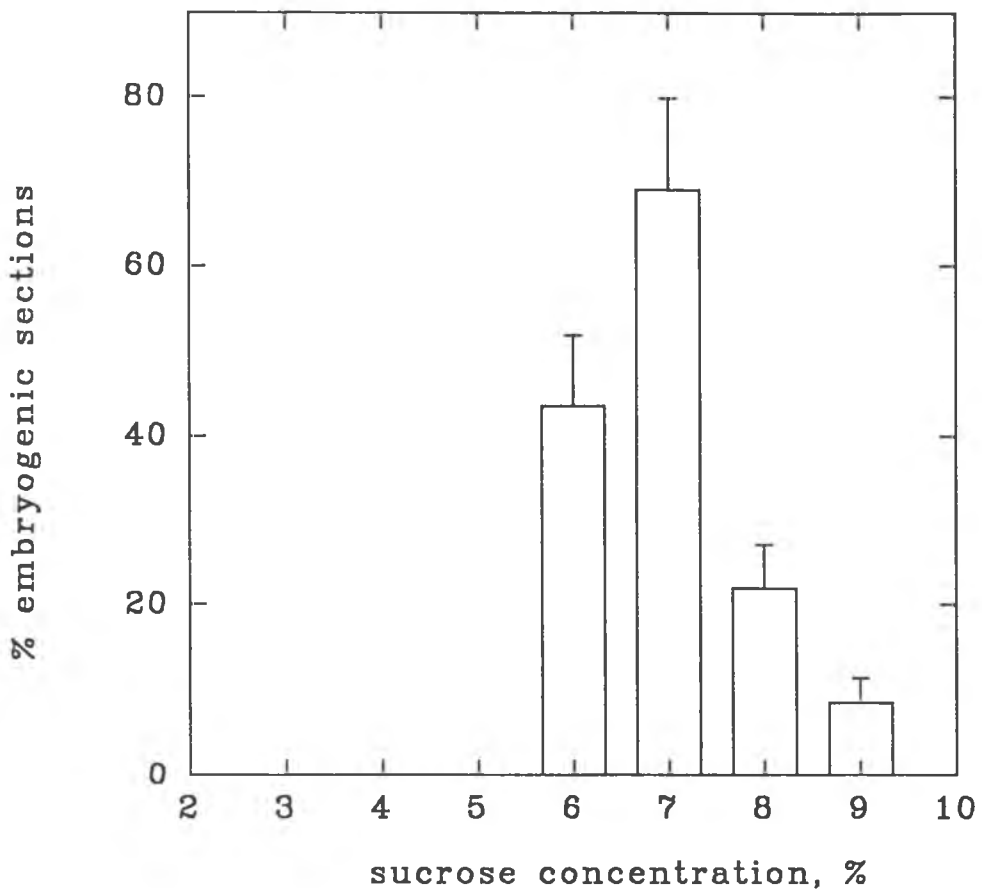

% embryogenic sections
0
20
40
60
80
2
3
4
5
6
7
8
9
10
sucrose concentration, %

Table 4.2. Embryogenic callus production by seedling tissues from four Hawaiian papaya cultivars grown on induction media containing various concentrations of 2,4-D and sucrose. Between 15 to 60 hypocotyl sections, one to eight cotyledons, or one to four shoots were cultured in each dish. A dish was scored as positive if it contained at least one embryogenic callus, regardless of the total number of explants. The data are means of at least two cultures per treatment (± standard error) and were recorded after two and a half to three and a half months of culture

CV	%S	2,4-D	HYPOCOTYLS	COTYLEDONS	SHOOTS
K	3	0.0	0.0	N	N
K	3	2.3	0.0	N	N
K	3	4.5	0.0	N	14.3±14.3
K	3	22.6	33.3±33.3	N	N
K	3	45.2	100.0	N	9.1±9.1
K	3	67.9	100.0	N	N
K	3	113.1	100.0	N	N
K	6	0.0	0.0	0.0	0.0
K	6	2.3	100.0	0.0	0.0
K	6	4.5	100.0	36.4±15.2	5.0±5.0
K	6	22.6	100.0	50.0±28.9	0.0
K	6	45.2	100.0	41.7±14.9	15.4±15.4
K	6	67.9	100.0	0.0	0.0
K	6	113.1	100.0	100.0	N
K	9	0.0	N	N	N
K	9	2.3	N	N	N
K	9	4.5	100.0	N	0.0
K	9	22.6	N	N	N
K	9	45.2	N	N	N
K	9	67.9	N	N	N
K	9	113.1	N	N	N
SR	3	0.0	0.0	0.0	0.0
SR	3	2.3	0.0	0	0.0
SR	3	4.5	N	N	N
SR	3	22.6	N	N	N
SR	3	45.2	0.0	0	25.0±25.0
SR	3	67.9	N	N	N
SR	3	113.1	0.0	50.0±28.9	0.0
SR	6	0.0	0.0	0.0	0.0
SR	6	2.3	53.8±14.4	15.4±10.4	12.5±12.5
SR	6	4.5	100.0	0.0	N
SR	6	22.6	50.0±28.9	0.0	50.0±28.9
SR	6	45.2	50.0±28.9	0.0	0.0
SR	6	67.9	0.0	0.0	N
SR	6	113.1	0.0	0.0	0.0

Table 4.2. (Continued) Embryogenic callus production by seedling tissues from four Hawaiian papaya cultivars grown on induction media containing various concentrations of 2,4-D and sucrose

CV	%S	2,4-D	HYPOCOTYLS	COTYLEDONS	SHOOTS
SR	9	0.0	50.0±28.9	0.0	0.0
SR	9	2.3	66.7±33.3	33.3±33.3	0.0
SR	9	4.5	N	N	N
SR	9	22.6	N	N	N
SR	9	45.2	50.0±28.9	0.0	0.0
SR	9	67.9	N	N	N
SR	9	113.1	0.0	0.0	0.0
SS	3	0.0	0.0	0.0	0.0
SS	3	2.3	0.0	0.0	33.3±33.3
SS	3	4.5	0.0	0.0	0.0
SS	3	22.6	33.3±33.3	33.3±33.3	50.0±28.9
SS	3	45.2	N	N	N
SS	3	67.9	N	N	N
SS	3	113.1	N	N	N
SS	6	0.0	0.0	0.0	0.0
SS	6	2.3	57.1±20.2	25.0±25.0	0.0
SS	6	4.5	62.5±18.3	0.0	11.1±11.1
SS	6	22.6	83.3±16.7	40.0±24.5	25.0±25.0
SS	6	45.2	100.0	0.0	N
SS	6	67.9	100.0	0.0	N
SS	6	113.1	50.0±28.9	0.0	N
SS	9	0.0	0.0	0.0	0.0
SS	9	2.3	0.0	0.0	0.0
SS	9	4.5	33.3±33.3	0.0	0.0
SS	9	22.6	66.7±33.3	33.3±33.3	33.3±33.3
SS	9	45.2	N	N	N
SS	9	67.9	N	N	N
SS	9	113.1	N	N	N
W	3	0.0	0.0	0.0	0.0
W	3	2.3	66.7±33.3	33.3±33.3	0.0
W	3	4.5	N	33.3±33.3	N
W	3	22.6	N	0.0	N
W	3	45.2	0.0	N	0.0
W	3	67.9	N	N	N
W	3	113.1	0.0	N	0.0
W	6	0.0	0.0	0.0	0.0
W	6	2.3	0.0	0.0	N
W	6	4.5	N	0.0	N
W	6	22.6	N	0.0	N
W	6	45.2	0.0	N	N

Table 4.2. (Continued) Embryogenic callus production by seedling tissues from four Hawaiian papaya cultivars grown on induction media containing various concentrations of 2,4-D and sucrose

CV	%S	2,4-D	HYPOCOTYLS	COTYLEDONS	SHOOTS
W	6	67.9	N	N	N
W	6	113.1	0.0	N	0.0
W	9	0.0	0.0	0.0	0.0
W	9	2.3	0.0	0.0	N
W	9	4.5	N	0.0	N
W	9	22.6	N	0.0	N
W	9	45.2	0.0	N	0.0
W	9	67.9	N	N	N
W	9	113.1	0.0	N	N

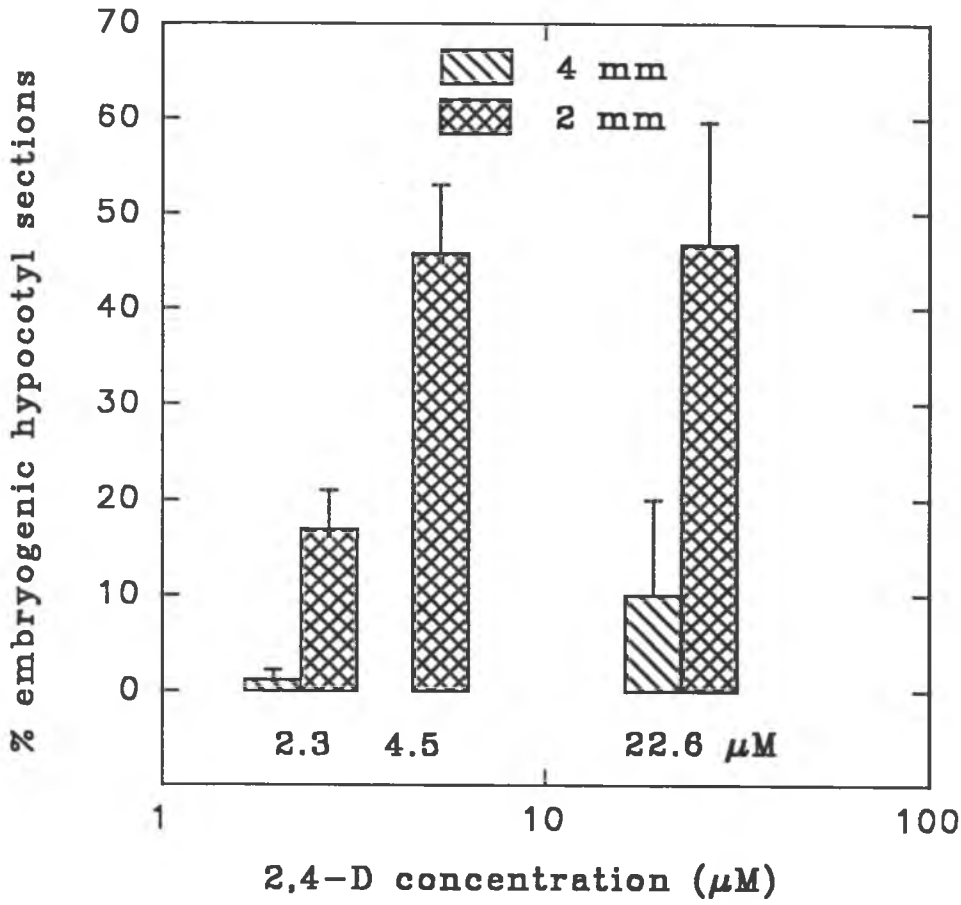
CV = cultivar
%S = sucrose concentration
2,4-D = μM 2,4-dichlorophenoxyacetic acid
K = 'Kapoho'
SR = 'Sunrise'
SS = 'Sunset'
W = 'Waimanalo'
N = no data or unreplicated dishes

Table 4.3. Embryogenic papaya seedling root cultures induced with 2,4-D in 1/2MS media containing 6% sucrose. The data are means ± one standard error from at least three petri dishes per treatment. Each sample dish contained one or two root masses

CV	2,4-D	TD	%Embryogenic samples
K	2.3	0.045	35.7±13.3
SS	2.3	0.045	40.0±24.5
K	4.5	0.0	100.0
SS	4.5	0.0	25.0±25.0
SS	4.5	0.023	66.7±33.3
K	4.5	0.09	77.8±14.7
SS	4.5	0.09	0.0

CV = cultivar
2,4-D = μM 2,4-dichlorophenoxyacetic acid
TD = μM thidiazuron
K = 'Kapoho'
SS = 'Sunset'

Fig. 4.5. Effect of hypocotyl length on induction of somatic embryogenesis after two and half to three and half months of culture. Mixtures of 'Kapoho' and 'Sunrise' were compared. Data are means of at least three replicates.


70
60
50
40
30
20
10
0
% embryogenic hypocotyl sections
4 mm
2 mm
2.3
4.5
22.6 μM
1
10
100
2,4-D concentration (μM)

All four Hawaiian hermaphrodite cultivars, 'Kapoho', 'Sunrise', 'Sunset', and 'Waimanalo', produced embryogenic hypocotyl calli on induction media supplemented with 3, 6, or 9% sucrose and 2,4-D. Optimal 2,4-D concentration was determined for 'Kapoho', 'Sunrise', and 'Sunset'; however, 'Waimanalo' requires further testing with treatments comparable in number to the 'Kapoho' experiments. The data support observations by Kamada and Harada (1979a, 1979b) and Ammirato (1983) that indicate that the growth regulator 2,4-D is required to induce embryogenic calli from explants. Optimal osmoticum concentration in an induction medium is of importance as evidenced by the response of 'Kapoho' to sucrose concentrations in a range from 3 to 9% (Fig. 4.4).

4.3.3 EMBRYOGENIC CALLI FROM OTHER EXPLANTS

Cotyledons, roots, and shoots from the seedlings grown for hypocotyl explants were also observed for embryogenesis. Media containing 2,4-D induced embryogenesis in the three types of tissues; however, embryogenesis in hypocotyl sections is more efficient and reliable, especially for the cultivar 'Kapoho' (Table 4.2).

4.3.3.1 COTYLEDONS

Prior to finding that 2,4-D was necessary for embryogenesis in the culture protocol, an attempt was made to obtain embryogenic calli from cotyledons using other growth regulators. Cotyledons cultured on media containing picloram and BA produced somatic embryos at extremely low frequencies. Litz et al. (1983) reported shoot organogenesis from cotyledon explants grown on a range of NAA and BA concentrations, but those results were not reproduced with the Hawaiian cultivars.

Fifteen combinations of sucrose and 2,4-D in induction media were plated with one to eight cotyledons from two of the four cultivars (Table 4.2). Sucrose concentrations used were 3, 6, and 9%, and 2,4-D ranged from 0 to 113.1 μM. Every cultivar was not represented in all treatments. No definite pattern of response could be discerned, since the data were not complete. 'Kapoho' was plated only on media containing 6% sucrose. From the limited data, it appears that 'Kapoho' cotyledons, like the hypocotyls, can form embryogenic callus in a wide range of 2,4-D concentrations, whereas 'Sunset', 'Sunrise', and 'Waimanalo' are less responsive (Table 4.2). 'Kapoho' cotyledon explants were plated either whole or sliced, but no difference was observed between treatments.

4.3.3.2 SHOOT APICES

Calli that grew from apical tissues cultured with cotyledons and hypocotyl sections, also developed embryogenic calli on media containing 2,4-D (Table 4.2). The culture media suitable for hypocotyls and cotyledons were also suitable for inducing embryogenic calli from seedling shoots. 'Kapoho' shoots produced embryogenic calli in nearly every medium on which they were cultured (Table 4.2), reflecting again the the positive embryogenic response of this cultivar in a wide range of 2,4-D concentrations. Embryogenic calli from 'Sunrise' and 'Sunset' were generated on 2.3 to 45.2 μM 2,4-D-containing media, but no pattern of response was obvious. The data show that seedling shoots of 'Kapoho', 'Sunrise', and 'Sunset' are capable of forming embryogenic callus, but in terms of amounts of embryogenic tissues generated per seedling, hypocotyls are more productive (Table 4.2).

4.3.3.3 ROOTS

Seedling root masses from two cultivars were cultured on several media. Unlike Chen et al. (1987) who cultured 1-cm root sections without apices, we plated the entire root mass cut off at the base of the hypocotyl. The cultivars 'Kapoho' and 'Sunset' produced embryogenic calli on the

apices of intact roots cultured on media containing 2,4-D (Table 4.3). The calli were visible after one month of culture on induction media containing 2,4-D with or without 0.023 to 0.045 μM thidiazuron (Table 4.3). Thidiazuron was apparently inhibitory to embryogenesis in 'Sunset' roots at the 0.09 μM concentration with 4.5 μM 2,4-D, whereas 'Kapoho' developed at least one embryogenic root apex after eight weeks on all media containing 2,4-D and TD (Table 4.3). Roots developed wet-looking calli that were white or golden brown in color. Shiny, undulating, light green structures developed at the tips of roots, and upon removal of the tissues to maturation medium, the structures differentiated into shoots. Somatic embryos produced on induction media containing 2,4-D alone resembled those initiated from hypocotyl sections and from immature zygotic embryos (Chapter 3). Chen et al. (1987) reported embryogenic calli from root sections whereas in our experiments with whole root masses, the apices of roots were the only tissues that produced embryogenic calli. Various 1/2MS organics media containing picloram, NAA, 2iP, and/or thidiazuron (Table 4.1) did not induce embryogenesis in roots. In this protocol, 2,4-D was essential for embryogenic callus initiation from seedling root apices.

The overall results from the observations of seedling tissues show that cotyledons, shoot apices, and root masses from ten-day-old seedlings can develop embryogenic calli on

the same media that induced embryogenic hypocotyl calli. However, hypocotyls are, by far, the most efficient producers of embryogenic calli, both in terms of frequency of occurrence of cultures (Table 4.2) and by volume of calli generated (Fig. 4.1E).

4.3.4 SOMATIC EMBRYO MATURATION, GERMINATION, AND SHOOT MICROPROPAGATION

Embryogenic calli developed into somatic embryos within one month after tranfer to maturation medium. They continued to develop and enlarge, especially with monthly subculture, but rarely germinated. Germination occurred on MS medium between one and four weeks after subculture. Some somatic embryos were misshapen and abnormally large. After the embryos germinated, green shoots were produced within one or two months. They could be removed and propagated when the stems were about 1-cm tall. Shoot tips from germinated embryos formed callus and rooted in MS medium with IBA in one to four weeks. Once roots were initiated, the shoots were transferred to MSV where the plants grew vigorously. Shoot cuttings taken from these vigorously growing plants were the fastest to develop roots (in one or two weeks). Cuttings from plants that ceased rapid growth did not root readily, nor did leafless cuttings as observed by Miller and Drew (1990). Shoots that were recalcitrant to

grow and root, when placed into MPII medium, resumed active growth, and new shoots that developed could be excised and rooted. When the plants were about 3-cm tall, they were transferred to potting soil, acclimated to laboratory or greenhouse conditions, and planted in the field. Survival was nearly 100% if plants were well-rooted and not contaminated with fungi or bacteria prior to potting.

High frequency somatic embryogenesis, demonstrated earlier in two types of papaya tissues, seedling roots (Chen et al. 1987) and immature zygotic embryos (Chapter 3), is reliably produced from seedling hypocotyl sections of four Hawaiian hermaphrodite cultivars. Somatic embryos may be less convenient to recover from immature zygotic embryos than from seedling tissues in locations where young papaya fruits are not readily available. They were produced more quickly from zygotic embryos than from hypocotyl calli, appearing after three to six weeks on zygotic embryos (Chapter 3) compared to four to ten weeks on hypocotyl sections (this report) and three months on root explants (Chen et al. 1987). The shorter period of time that the zygotic embryo tissues were exposed to 2,4-D may increase the percentage of somatic embryos that germinate normally, and may also decrease the incidence of abnormalities that may arise from the 2,4-D treatment (Larkin and Scowcroft 1981, Orton 1985).

Recently, cultures of immature zygotic embryos pre-treated with 2,4-D and hypocotyl- and zygote-derived somatic embryos had been subjected to two transformation methodologies, *Agrobacterium*- (unpublished results) and particle gun-mediated gene transfer (Chapter 5). Evidence for stable transformation following particle bombardment with chimeric genes for neomycin phosphotransferase (NPTII) and ß-glucuronidase (GUS) has been reported (Chapter 5). *Agrobacterium*-vectored transformation has also been observed in somatic embryo cultures of papaya transformed with constructs containing the GUS gene, NPTII gene, and a coat protein gene for papaya ringspot virus (unpublished results).

CHAPTER 5. STABLE TRANSFORMATION OF PAPAYA VIA MICROPROJECTILE BOMBARDMENT

5.1 INTRODUCTION

Stable transformation of crop plants has been achieved through the use of several DNA transfer technologies. The most widely employed approach, involving *Agrobacterium* vectors (Caplan et al. 1983, Klee et al. 1987, Kuhlemeier et al. 1987) is limited by host range specificity that excludes most monocotyledonous species. Other transformation methods, including microinjection, direct DNA uptake, and electroporation, are effective only with protoplast cultures; however, regeneration may be difficult, and for some species regeneration procedures do not presently exist (Paszkowski et al. 1984, Cocking and Davey 1987). Problems of vector specificity and difficult regeneration have been reportedly overcome in a few special cases, e.g., transformation of barley by injection of DNA into young floral tillers (de la Peña et al. 1987), transformation of rice by the pollen-tube pathway (Luo and Wu 1988), and transformation of imbibing *Arabidopsis* seed in the presence of *Agrobacterium* (Feldmann and Marks 1987).

A more generally applicable transformation method that also circumvents these obstacles is microprojectile bombardment (Klein et al. 1987, Sanford 1988). Initial

results with this technique revealed transient expression of gene constructs in target tissues (Klein et al. 1988a, Klein et al. 1988b, Wang et al. 1988). More recent publications showed that stable integration is also possible (McCabe et al. 1988, Klein et al. 1988c, Klein et al. 1989). We report the use of microprojectile bombardment to stably transform three types of papaya tissues, which subsequently produced somatic embryos and plants on selective medium.

5.2 MATERIALS AND METHODS

5.2.1 PLANT MATERIAL AND TISSUE CULTURES

Embryogenic papaya (*Carica papaya* L.) cultures were initiated from zygotic embryos and seedling hypocotyl sections (Chapters 3 and 4). Immature zygotic embryos were excised from the cultivars 'Sunset' and 'Kapoho' at 90 to 105 days after pollination, and one cotyledon was removed from each embryo to expose the apical meristem. Zygotic embryos were plated on induction medium which consisted of half-strength MS (Murashige and Skoog 1962), 2.7 mM glutamine, 0.3 mM myo-inositol, full-strength MS vitamins, 6% sucrose, 45.2 μM 2,4-D, and 1% Sigma A1296 agar, pH 5.8. Four- to 23-day-old cultures of zygotic embryos provided one tissue type for bombardment. One week prior to bombardment, the embryos were transferred to 6-cm petri dishes in groups

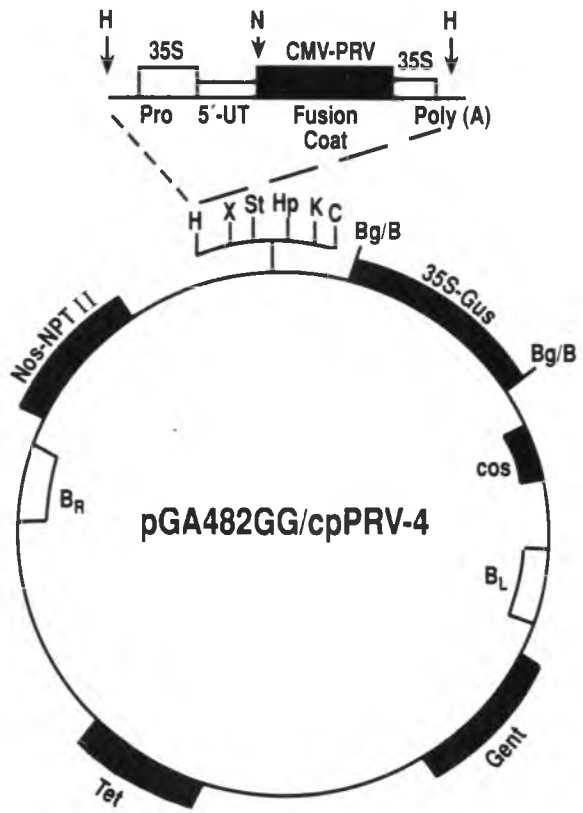
of 40 to 100 per dish. Embryos were oriented with apical meristems exposed and radicles embedded in induction medium solidified with 1.5% agar. The higher agar concentration reduced splatter and culture disruption during bombardment. Embryos were arranged in a 2.5-cm-diameter "doughnut" configuration to avoid blast damage from microprojectiles in the central 1.0 cm of the target. Hypocotyls from 14-day-old 'Kapoho' seedlings, grown on water agar (1%), were sliced into 2-mm sections and about 175 sections were plated directly into each 6-cm petri dish containing induction medium with 1.5% agar. One- to two-day-old cultures were bombarded. Six-month-old hypocotyl and zygotic embryo cultures, initiated as described above, grown in darkness at 27^{o}C and subcultured at irregular intervals on induction medium, yielded embryogenic callus and somatic embryos for the third type of culture bombarded. About 500 mg fw of tissue was plated onto each Whatman #2 filter paper on induction media. Two other media used in this study were maturation medium and micropropagation medium. Maturation medium consisted of 2,4-D-free induction medium. Regenerated plants were grown under 35 μmol m^{-2} sec^{-1} photosynthetically active radiation (PAR) in liquid micropropagation medium which consisted of MS, 0.6 mM myo-inositol, 3.0 μM thiamine·HCl, 2% sucrose, 0.5 μM kinetin, and 0.9 μM 6-benzylaminopurine, pH 5.8.

Thirteen petri dishes of hypocotyl segments, 26 dishes of four- to 23-day-old zygotic embryo cultures, and 38 dishes of embryogenic calli and somatic embryos were subjected to bombardment with DNA-coated microprojectiles. Cultures were transferred to fresh induction medium eight to ten days following bombardment. Those contaminated with bacteria were plated in the same medium with a supplement of 0.4 mM cefotaxime (Calbiochem, San Diego, CA). Contamination was believed to have originated from a few contaminated zygotic embryo cultures that were bombarded. Cultures were incubated at 27°C in darkness for three weeks. Subsequently, most cultures were transferred to maturation medium supplemented with 129 μM kanamycin sulfate (Sigma Chemical Co., St. Louis, MO), but about one-quarter of the cultures were retained on fresh induction medium containing 129 μM kanamycin sulfate. Cultures that were suspected of being contaminated were plated on maturation or induction medium containing either 1.2 mM carbenicillin or 60 μM rifampicin (Sigma Chemical Co.) Following four more weeks of culture, the kanamycin concentration was increased to 257 μM in both maturation and induction media. The cultures were not transferred again for eight weeks. The hypocotyl segment cultures, after five months on the initial selective medium, were maintained on induction medium containing 257 μM kanamycin. Thereafter, all cultures were subcultured monthly.

5.2.2 PLASMIDS AND DNA DELIVERY

The plasmids pGA482GG (17 kb), pGA482GG/cpPRV-4 (18.6 kb), and pGA482GG/cpPRV-19-5 (18.6 kb) are derivatives of the *Agrobacterium* binary vector pGA482 (An 1986). Two of the plasmids have been modified to contain chimeric genes for the coat protein of the mild mutant strain HA 5-1 of papaya ringspot virus (PRV) (Yeh and Gonsalves 1984a) and for a bacterial GUS gene (Jefferson 1987), in addition to the NPTII gene of pGA482. Coding sequences for the PRV coat protein gene were obtained from the pUC9 clone pPRV117. The GUS reporter gene was inserted between CaMV 35S promoter and *nos* 3′ termination sequences. The plasmids pGA482GG/cpPRV-4 and pGA482GG/cp19-5-2 differ in arrangement of leader sequences in the chimeric PRV coat protein gene, and their construction is described in Fig. 5.1. The NPTII gene is flanked by *nos* promoter and termination sequences. A pGA482GG plasmid lacking the PRV coat protein gene was used as a control for plasmids pGA482GG/cpPRV-4 and pGA482GG/cpPRV-19-5. Plasmid DNAs were isolated as described by Maniatis et al. (1982) followed by banding in an ethidium bromide CsCl gradient.

Fig. 5.1. Plasmid vectors used for coating the microprojectiles were all derived from the *Agrobacterium* binary vector pGA482 (An 1986) which contains the T-DNA-derived border fragments B_R and B_L, the *cos* site for the bacterial phage lambda, a restriction enzyme polylinker, and the NOS-NPTII fusion gene. Addition of the bacterial gentamicin gene (Gent) outside of the T-DNA region has been described by Chee et al. (1989). The presence of this gentamicin has no function when used by microprojectile bombardment; it is useful for plasmid transfers into *Agrobacterium*. The bacterial GUS gene, constructed by Jefferson (1987), was cloned into the vector by removing the GUS gene via a partial *Bam*HI digestion and cloning this 3.0 kb fragment into the *Bgl*II site of the polylinker of pGA482 (Quemada, Slightom, unpublished data). Expression cassettes PRV-4 and PRV-19 (H. Quemada, J. Slightom, unpublished data) were designed for cloning into the *Hin*dIII site of the polylinker; only the vector of pGA482GG/cpPRV-4 is shown since pGA482GG/cpPRV-19-5 differs only in the linkage of the 5′-untranslated region (5′-UT) with the PRV coat protein gene (H. Quemada, J. Slightom, unpublished data). Restriction enzyme sites are: Bg/B, nondigestable fusion of *Bgl*II and *Bam*HI; C, *Cla*I; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; N, *Nco*I; St, *Stu*I; and X, *Xho*I.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H
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CMV-PRV
35S
Pro
5'-UT
Fusion Coat
Poly (A)
H
X
St
Hp
K
C
Bg/B
35S-Gus
Bg/B
cos
Nos-NPT II
B_R
pGA482GG/cpPRV-4
B_L
Gent
Tet

Plasmid DNA was transferred to tissues using the particle gun described by Klein et al. (1988a). Plasmid DNA was precipitated from ethanol, centrifuged at 15,000 rpm for 15 minutes and vacuum dried for ten minutes. The DNA was suspended in deionized water at a concentration of 1.0 $\mu g\ \mu l^{-1}$. Particle coating and bombardment were essentially as reported by Klein et al. (1988a, 1988b). Tungsten particles (Sylvania M10) were suspended in distilled water and 25 μl aliquots were pipetted into 1.5 ml microfuge tubes, followed by aliquots of 2.5 μl DNA, 25 μl $CaCl_2$ (2.5 M) and 10 μl spermidine (0.1 M-free base). The preparations were vortexed and allowed to settle for ten minutes. They were pulsed briefly in a microcentrifuge and 45 μl of supernatant was discarded. Immediately prior to loading the coated particles onto macroprojectiles, the microfuge tubes were sonicated briefly or finger vortexed to suspend the particles. Two μl of suspension were delivered per bombardment and each plate was bombarded three times except for two hypocotyl plates bombarded four times.

5.2.3 GUS HISTOCHEMICAL ASSAY

Three weeks following particle bombardment, and at irregular intervals thereafter, papaya tissue cultures were examined for GUS expression using the histochemical assay

(Jefferson et al. 1986, Jefferson 1987). Samples of zygotic embryos, hypocotyl segments, or somatic embryos and calli having fresh weights of about 100 mg each were transferred to 1.5-ml microfuge tubes and treated with 400 μl of 1.0 mM X-gluc in 200 mM sodium phosphate buffer, pH 7. Color development was recorded after one to 12 hours of incubation at 37^{o}C. Four months following particle bombardment, segments of putative transformants that grew on the selective media could be assayed for GUS expression without sacrificing the entire individual. Slices of cotyledons, hypocotyls, leaves, and roots or portions of the total mass of somatic embryos were incubated in X-gluc as described above.

5.2.4 NPTII ASSAY

Six months following particle bombardment, somatic embryos which grew on media containing kanamycin were assayed for NPTII expression following the protocol of McDonnell et al. (1987). Fresh weights of tissue samples ranged from less than 10 mg to 220 mg. In our first assay, the small amount of tissue available limited extract concentrations to less than 1.0 mg μl^{-1} of extraction buffer. In subsequent assays, samples weighing about 150 mg each were ground in sufficient extraction buffer to yield extract concentrations of 1.0 mg μl^{-1} of buffer. All other

aspects of the procedure described by McDonnell et al. (1987) were followed. One minute counts on a Beckman model 1801 scintillation counter were taken and autoradiographs were exposed for 48 hours to ten days. Expression results were based on scintillation counts and were recorded as sample counts per minute (cpm) per μg protein. Protein was determined by the Bradford method (1976).

5.3 RESULTS AND DISCUSSION

5.3.1 CULTURES

A total of 77 culture plates of embryogenic zygotic embryos (Fig. 5.2A), embryogenic calli and somatic embryos derived from hypocotyls and zygotic embryos (Fig. 5.2B) and explanted hypocotyl segments were bombarded with DNA-coated microprojectiles. DNA used for coating the microprojectiles consisted of either pGA482GG, pGA482GG/cpPRV-4, or pGA482GG/cp19-5-2 (Fig. 5.1). Seven of the culture plates were badly contaminated and had to be discarded. Nearly half of the 70 remaining culture plates had small bacterial colonies growing on tissues and were treated with antibiotic-supplemented media. Based on the expression of scorable and/or selectable marker genes, ten out of the 70 culture plates gave rise to 15 different transgenic somatic embryo isolates. Ten of the isolates were recovered from

2360 surviving bombarded zygotic embryos for a percentage yield of 0.42. Five of the isolates grew from about 1.7 g fw of bombarded somatic embryo cultures. It was not possible to quantify the somatic embryo cultures except by approximate fresh weight. A total of about 19 g fw of somatic embryos and calli were bombarded.

5.3.2 GROWTH ON KANAMYCIN

Cultures were allowed to recover from bombardment for three to five weeks before transfer to selective media containing 129 μM kanamycin. This level of kanamycin, while adequate to suppress induction of embryogenesis in freshly explanted tissues, was not sufficient to completely inhibit growth of somatic embryos that had developed prior to being plated on selective medium. Consequently, after four to six weeks, the kanamycin concentration was doubled for most of the cultures. The higher level effectively stopped growth of untransformed papaya tissues in the bombarded cultures (data not shown).

Four months after bombardment, seven putative transgenic isolates were growing vigorously on media containing kanamycin. In one case, culture 62-2, the resistant somatic embryo cluster was clearly chimeric, having a green, developing sector set off sharply from the pale ivory-colored embryonic tissues. In some cases, growth

of the putative transformants was rapid. One month after these isolates were observed and subcultured onto fresh maturation medium containing 257 μM kanamycin, we were able to assay parts of the plants or portions of the somatic embryo clusters to confirm that transformation had occurred.

5.3.3 GUS ASSAY

Starting three weeks after bombardment, we began to assay the cultures for GUS expression using X-gluc substrate. We tested 240 zygotic embryos, or about 10% of the total number bombarded, and observed 47 dark blue, GUS-positive spots on 23 zygotic embryos. In 29 of these cases, GUS expressing cells were located in the cotyledons and therefore lacked the potential for plant regeneration in our tissue culture system. The remaining 18 GUS spots were observed in the swollen, embryogenic apical domes of the zygotic embryos (Fig. 5.2C). These tissues had the potential to give rise to transformed plants. In apical domes assayed one month after bombardment, blue spots became visible after ten hours of incubation. However, when embryogenic tissues derived from apical domes were assayed five months after bombardment, GUS expression was evident after only 50 minutes of incubation, presumably due to the response of a larger number of transformed cells.

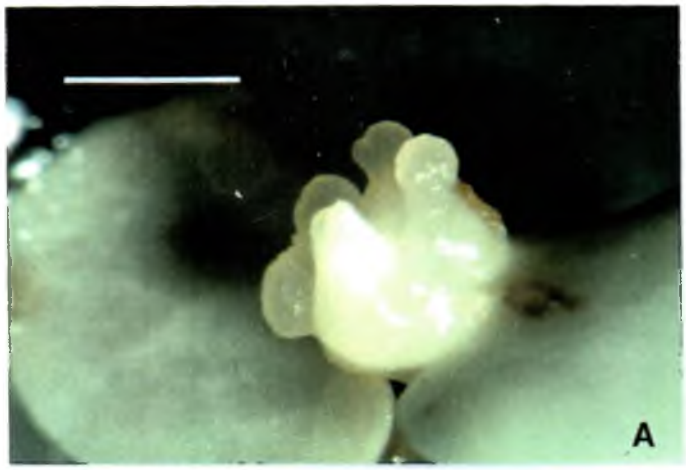
Bombardment of somatic embryo preparations (Fig. 5.2B) and hypocotyl segments was not as effective in producing GUS-positive spots as bombardment of zygotic embryos. Assaying about the same amount of bombarded surface area of somatic embryos and embryogenic calli yielded only eight dark blue spots compared with 47 spots on zygotic embryos. These spots were observed on hypocotyls and cotyledons of somatic embryos (Fig. 5.2D) and on clusters of globular somatic embryos. Hypocotyl segments were not assayed extensively because they showed only one GUS-positive cell in initial tests conducted soon after bombardment. However, six months following bombardment, one embryogenic callus piece derived from a hypocotyl segment produced a strong GUS-positive response, suggesting that hypocotyl tissue may also have potential for transformation with the particle gun.

The nature of GUS staining differed considerably (Fig. 5.2C to 5.2F) in tissues from different transformation events. Some somatic embryos appeared uniformly dark blue, while others were pale, as if only the epidermis had been transformed. Globular somatic embryos of isolate 41-1 showed an intense blue GUS response (Fig. 5.2F), while cotyledons of more mature somatic embryos from this isolate showed only pale blue GUS expression.

5.3.4 NPTII ASSAY

The results of the NPTII assay confirmed that ten of the papaya somatic embryos showing uninhibited growth on selective medium four to eight months after particle bombardment were indeed transformed. A total of ten somatic embryo isolates and five regenerated plant isolates from ten bombarded plates were tentatively identified as transgenic based on growth on selective medium. Three of the ten NPTII-expressing somatic embryo isolates also showed GUS activity. Table 5.1 shows the correspondence between growth on kanamycin-containing media and expression of NPTII and GUS in putative transformed isolates. The discrepancy between our results and those of Klein et al. (1989), who reported that all isolates expressing NPTII also showed some degree of GUS expression, may be due to the larger sizes of our vectors (17 and 18.6 kb) compared to theirs (8.2 kb). Larger plasmids may be more subject to fragmentation during particle bombardment. Since NPTII and GUS chimeric genes are at opposite ends of our expression cassette in pGA482GG/PRV, random fragmentation would result in reduced linkage of these genes. The preponderance of NPTII expression is not surprising, since selection is based upon

Fig. 5.2. Papaya cultures are shown prior to and following bombardment. Transgenic somatic embryos and plants that resulted from the DNA transfer are shown. A) Zygotic embryo of 'Kapoho' papaya after induction of somatic embryos on apex between cotyledons. Scale = 1 mm. B) Somatic embryos of 'Kapoho' papaya initiated from hypocotyl segments cultured on induction medium. Scale = 1 mm. Cultures such as those in Fig 5.1A and 5.1B were bombarded with DNA-coated tungsten particles. C) Zygotic embryo that had proliferated a large mass of somatic embryos from its apical dome. Assayed three weeks following particle bombardment; a small, blue spot (arrow) indicated GUS activity. Scale = 1 mm.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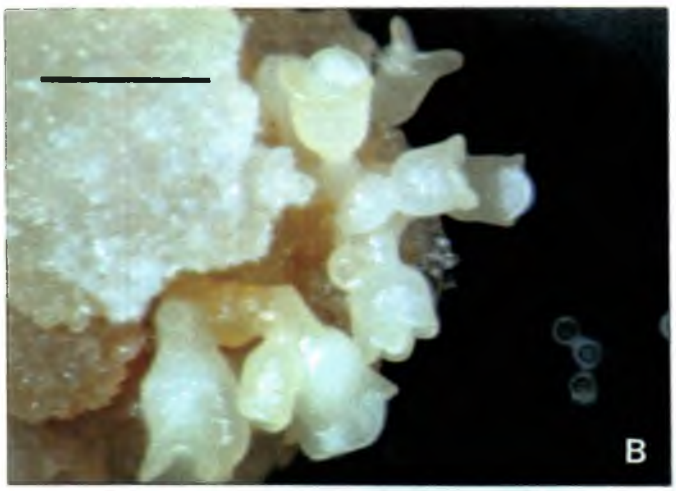
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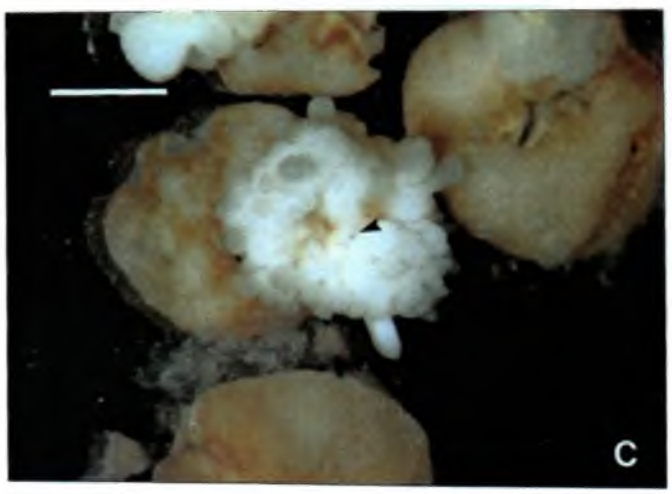
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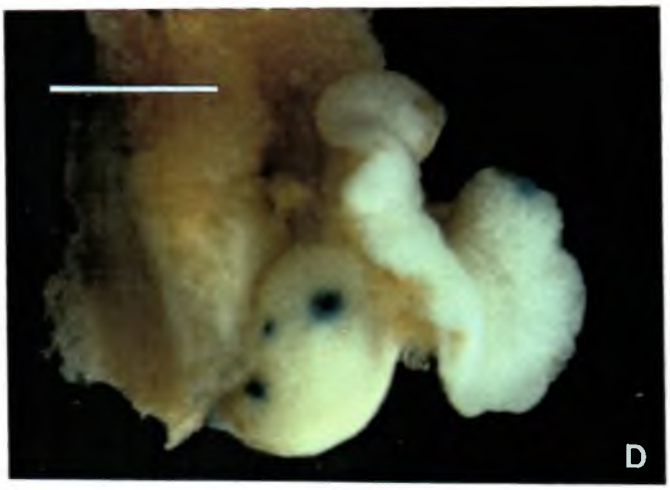
Fig. 5.2. (Continued) Papaya cultures are shown prior to and following bombardment. Transgenic somatic embryos and plants that resulted from the DNA transfer are shown. D) Somatic embryo exhibited three GUS-positive blue spots on the hypocotyl, one on the cotyledon. Assayed seven months after treatment (preliminary experiment). Scale = 1 mm. E) GUS-positive cells of the radicles of 'Sunset' somatic embryos, three months following particle bombardment, illustrates the possible chimeric nature of transgenic tissues. Scale = 1 mm. F) Four months after particle bombardment, putative transformants were observed on kanamycin-containing media. 'Kapoho' culture 41-1 showed strong GUS expression. Scale = 1 mm.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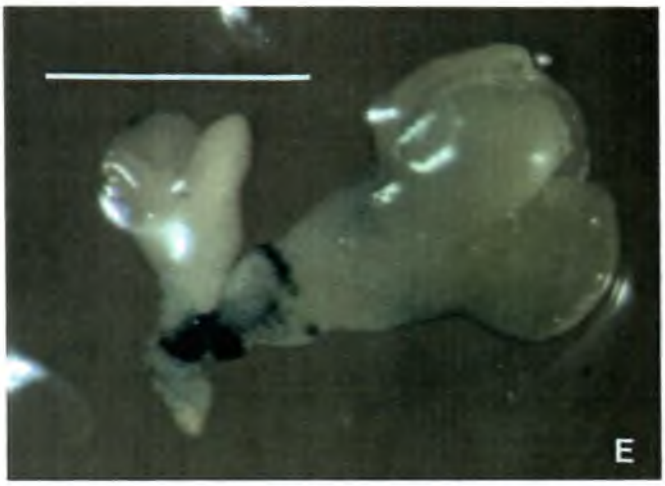
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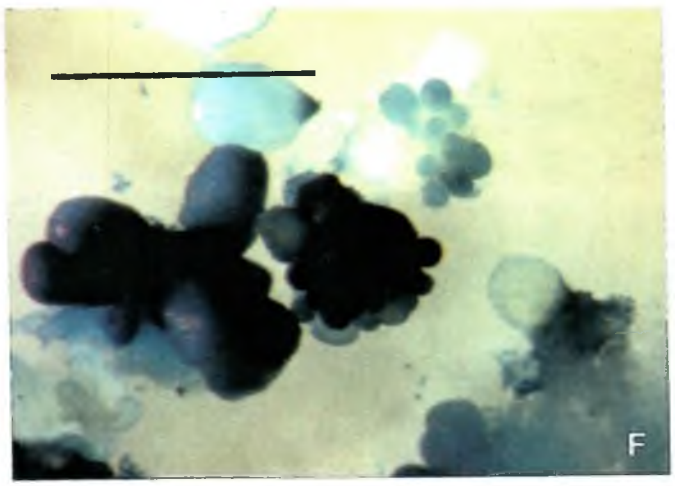
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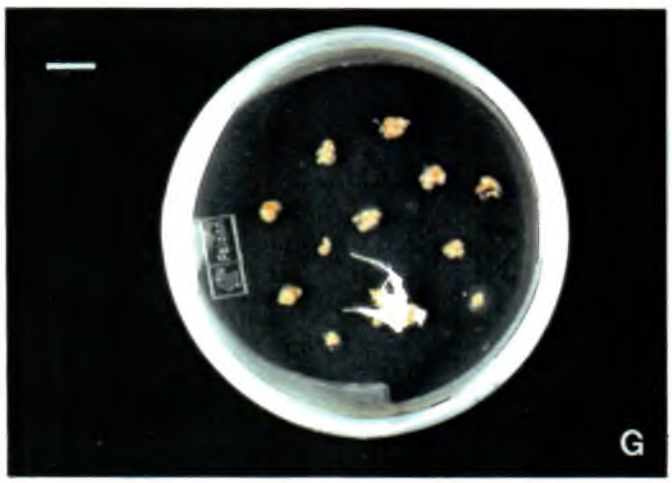
Fig. 5.2. (Continued) Papaya cultures are shown prior to and following bombardment. Transgenic somatic embryos and plants that resulted from the DNA transfer are shown. G) Kanamycin-resistant growth of somatic embryos budding off zygotic embryos was observed in cultures four months following particle bombardment. Scale = 1 cm. H) Leaves of 62-2 showed strong NPTII expression but no GUS expression. At the left are untransformed somatic embryos, at the right 'Sunset' 62-2, in micropropagation medium containing 257 μM kanamycin. Both somatic embryo cultures were about the same size when these cultures were initiated two months prior to being photographed. Scale = 1 cm.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H

Table 5.1. Scorable (GUS) and selectable (NPTII) marker genes in putative transgenic papaya isolates growing on selection medium (257 μM kanamycin)

Isolate No.	Tissue treated	Tissue assayed	GUS	NPTII	Growth on kan
Control	NA	SE	0	2.7±0.93[b]	0
Control	NA	leaf	0	1.1±0.21	0
9-1	H	SE	+++	ns	++
25-1[a]	SE	SE	0	5.3	+++
27-1[a]	SE	SE	+++	11.8±5.07	+++
27-2	SE	SE	+++	0.8	+++
29-1	SE	SE	+++	6.2±1.41	+++
40-1[a]	ZE	SE	0	24.4	+++
41-1[a]	ZE	SE	+++	4.5±0.92	+++
47-1	ZE	SE	0	18.4±7.59	++
50-1	ZE	SE	0	6.3	+++
57-1	ZE	SE	0	ns	+++
60-1	ZE	SE	+++	8.8	++
60-2	ZE	SE	0	3.8±2.58	++
62-1[a]	ZE	SE	0	51.0	+++
62-2[a]	ZE	SE	0	22.8	+++
62-2[a]	ZE	leaf	0	49.1±20.01	+++
62-3	ZE	SE	0	71.5	+

Control cultures were not bombarded with microprojectiles
H = hypocotyl sections, SE = somatic embryos, ZE = zygotic embryos, NA = not applicable, ns = not assayed
0 = no expression, + = slight, ++ = moderate, +++ = strong
[a]Isolates that have regenerated leafy shoots
[b]NPTII data values represent scintillation cpm/μg protein plus or minus one standard error for isolates assayed more than once. Values larger than control plus one standard error are considered NPTII-positive

All of the isolates listed in Table 5.1 are being grown and propagated for further testing. Some or most of them may be chimeras. Consequently, portions of each have been transferred to micropropagation medium containing 257 μM kanamycin to promote further axillary growth of transgenic sectors.

We have shown that particle bombardment resulted in stable expression of chimeric genes coding for NPTII and GUS in papaya. By regenerating transgenic papaya shoots, we have extended the work of Pang and Sanford (1988), who obtained transformed papaya leaf disk calli. Our work supports that of others who have reported transgenic plants from particle gun-mediated DNA transfer in rice, maize, and soybean. This work sets the stage for testing for expression of the third gene included in our constructs, PRV coat protein gene. The ultimate goal of this project is genetically engineered virus resistance as first described by Powell Abel et al. (1986).

CHAPTER 6. CHARACTERIZATION OF TRANSGENIC PAPAYA PLANTS FROM MICROPROJECTILE BOMBARDMENT

6.1 INTRODUCTION

Genetic transformation may help elucidate basic principles of gene function and control in plants (Schell 1987, Kuhlemeier et al. 1987) as well as enhance crop productivity with elegant new methods of solving problems like virus resistance (Powell Abel et al. 1986, Goodman et al. 1987, Grumet 1990, Beachy et al. 1990). Several methods for transforming plants have proven effective, for example, *Agrobacterium*-mediated transformation in dicots (Klee and Rogers 1989), direct DNA uptake in monocots (Potrykus 1990a, 1990b), microprojectile bombardment (Sanford 1990, Christou 1990), and microinjection (Neuhaus and Spangenberg 1990). Except for a few well-studied crops, efficient transformation protocols still need to be developed before specific genes useful for crop improvement can be routinely transferred and tested. "Model" crops, tobacco, maize, soybean, and rice, can be transformed by more than one method. For example, transgenic tobacco plants expressing foreign genes have been recovered from protoplast uptake of DNA (Paszkowski et al. 1984), co-cultivation of protoplasts with *Agrobacterium* (Fraley et al. 1985), co-cultivation of leaf disks with *Agrobacterium*

(Horsch et al. 1985), and microprojectile bombardment of calli and leaves (Klein et al. 1988c, Tomes et al. 1990). Transgenic maize plants have been regenerated after direct DNA uptake by protoplasts (Rhodes et al. 1989), bombardment of embryogenic calli (Gordon-Kamm et al. 1990, Fromm et al. 1990) and *Agrobacterium*-mediated gene transfer (Gould et al. 1991).

Papayas have little resistance to papaya ringspot virus (PRV) (Conover and Litz 1978). Thus, a transformation project was initiated to produce genetically engineered virus resistance by inserting the coat protein gene of a mild strain of PRV (Quemada et al. 1990) into the genome of commercial Hawaiian cultivars. Coat protein-mediated virus resistance was first shown to delay the onset of severe symptoms of tobacco mosaic virus (TMV) in tobacco plants transformed with the coat protein gene of TMV (Powell Abel et al. 1986). "Pathogen-derived" resistance (Sanford and Johnston 1985) has since proven to be effective in crops other than tobacco when transformed with coat protein genes; they are tomato (Cuozzo et al. 1988, Nelson et al. 1988, Tumer et al. 1987) and potato (Hemenway et al. 1988, Hoekema et al. 1989, Lawson et al. 1990).

Papaya leaf disks have been transformed with *Agrobacterium tumefaciens* (Pang and Sanford 1988), and evidence has been presented for transformation and plant regeneration following microprojectile bombardment of

embryogenic papaya cultures (Chapter 5). We now report on the molecular characterization of several transgenic isolates from the bombardment experiment and compare the results from three types of tissues that served as targets for the gene constructs.

6.2 MATERIALS AND METHODS

6.2.1 PLANT MATERIAL AND CULTURE CONDITIONS

Cultures of freshly explanted hypocotyl sections, embryogenic calli and somatic embryos, and pretreated 90- to 105-day-old zygotic embryos were prepared for particle bombardment as described (Chapter 5). Embryogenic calli and somatic embryos of Hawaiian papaya cultivar 'Kapoho' were produced from hypocotyl sections of 14-day-old seedlings as previously described (Chapter 4). Embryogenesis was induced in zygotic embryos of 'Kapoho' and 'Sunset' with 2,4-D (Chapter 3).

6.2.2 PLASMID CONSTRUCTS AND GENE DELIVERY

Chimeric genes for PRV coat protein and GUS were cloned into the polylinker site of pGA482 (An 1986) in constructs described previously (Chapter 5). Plasmid DNAs were isolated and purified by CsCl ethidium bromide

centrifugation (Maniatis et al. 1982), precipitated from ethanol, and loaded onto tungsten microprojectiles as described by Klein et al. (1988a). A majority of the cultures were bombarded three times with the biolistics device as described earlier (Chapter 5).

6.2.3 RECOVERY OF TRANSGENIC EMBRYOS AND PLANTS

After a three- to five-week recovery period on induction medium, most of the bombarded cultures were transferred to maturation medium containing 129 μM kanamycin. The concentration of the selective agent was doubled after one month and sustained at the higher concentration for the duration of selection (Chapter 5). Four months after bombardment, putative transgenic isolates were observed. Transgenic somatic embryos germinated on MS medium containing kanamycin, but selectively growing embryos isolated later in the experiment were germinated without kanamycin. MS medium consisted of MS salts, 0.6 mM myo-inositol, 1.2 μM thiamine·HCl, 3% sucrose, and 0.5% Sigma A1296 agar, pH 5.8.

Shoots or germinated embryos were micropropagated for rapid growth in liquid MPII medium (Lee 1987). Shoot tip cuttings 1.0-cm long were excised from micropropagated clusters, and one to ten shoots were rooted in 35 ml of MS agar medium containing 4.9 μM indole-3-butyric acid

(MS/IBA). Rooted plants were transferred to jars containing a 50/50 mixture of autoclaved vermiculite and liquid MS medium. Roots were also initiated on cuttings from unrooted shoots growing rapidly in MS/IBA medium or from rooted plants in vermiculite. Eight replicates of each isolate line were compared to determine if isolate lines differed in number of days to root on MS/IBA medium and if the medium on which the cutting source was grown affected the number of days for rooting to occur. Data were collected on the length of time required for root initiation and the percentage of cultures that rooted in a two-month interval. The differences between means from eight replicates were compared with t or F test statistics in analyses of variance for replication and isolate lines. Plants with well-developed roots were rinsed of medium, potted in commercial potting soil, and covered with plastic bags for a week or two. The bags were perforated, and the holes were gradually enlarged to acclimate the plants to greenhouse conditions.

6.2.4 GUS HISTOCHEMICAL ASSAY

Leaves from germinated somatic embryos with apparent resistance to kanamycin were sliced and incubated overnight at 37^{o}C in filter-sterilized 0.5 mM 5-bromo-4-chloro-3-indolyl-ß-glucuronide (X-gluc, Jersey Lab Supply, NJ) according to the method of Jefferson (1987). Leaf tissues

were cleared of chlorophyll with several changes of 95% ethanol to enhance visualization of the blue precipitate. Isolates that initially tested negative for GUS were re-tested at least five times before they were scored.

6.2.5 DNA EXTRACTION

DNA was extracted using a modification of the "CTAB" method of Dellaporta et al. (1983). Leaves from putative transgenic plants in tissue culture were freeze-dried prior to DNA extraction. Between 50 and 700 mg dry weight (dw) of tissues were pulverized with autoclaved mortars, pestles, and silica sand. Regardless of the dry weight, each pulverized sample was extracted with 12 ml of extraction buffer (65^{o}C) in 50-ml disposable centrifuge tubes. Extraction buffer consisted of 0.1 M Tris-HCl (pH 8), 0.05 M EDTA (pH 8), 0.5 M NaCl, 10 mM 2-mercaptoethanol, and 6% SDS. The buffer was made by mixing the following stock solutions:

2.0 M Tris-HCl (pH 8)	7.5 ml
0.5 M EDTA (pH 8)	15.0 ml
5.0 M NaCl	15.0 ml
2-mercaptoethanol	300.0 μl

Volume made up to 150 ml with water, 10 ml of 20% SDS added, buffer heated to 65^{o}C

The samples were extracted for ten minutes at 65^{o}C. Cold 5.0 M potassium acetate (3.0 M potassium, 5.0 M acetate,

Maniatis et al. 1982) was added, 5.0 ml per tube, to precipitate proteins and polysaccharides. The mixtures were incubated in an ice bath for 20 minutes, transferred to sterile 50-ml Oakridge centrifuge tubes, and centrifuged at 10,000 rpm for 20 minutes. The supernatant was transferred to clean Oakridge tubes, and 0.8 volumes (13 ml) of isopropanol were added to each tube to precipitate nucleic acids. The mixtures were incubated for 30 minutes in an ice bath and centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded, and the pellet was washed three times with 5.0 ml 70% ethanol. The pellet was dissolved in minimal TE (10 mM Tris-HCl, pH 8; 1.0 mM EDTA, pH 8). About 600 μl of TE was used for a 700 mg dw leaf sample. A final NaCl concentration of 0.7 M was achieved by addition of 5.0 M NaCl to the dissolved samples. An equivalent volume of 1% cetyl trimethylammonium bromide (CTAB, Sigma Chemical Co.) was added. The mixtures were incubated at room temperature for 30 minutes and centrifuged at 10,000 rpm for ten minutes. The pellets were washed with 76% ethanol containing 0.2 M sodium acetate and dried under vacuum. The pellets were dissolved a second time in minimal TE (about 900 μl), transferred to 1.5-ml microfuge tubes, and centrifuged for ten minutes at 14,000 rpm to remove undissolved material. The supernatant was removed to clean microfuge tubes, nucleic acids were precipitated with 0.8 volumes of isopropanol, dried under vacuum, dissolved a

third time in 100 to 200 μl TE, and each tube was treated with 2000 units of RNase (DNase-free, #1119 915, Boehringer-Mannheim, Indianapolis, IN) for two hours at 37^{o}C. The quality and quantity of DNA was determined with a Beckman DU-50 spectrophotometer. Two μl of DNA from each sample were mixed with 750 μl water, optical density was determined at A_{260} and A_{280}, and the A_{260}/A_{280} ratios were used to determine DNA quality. DNA quality was also assessed by determining if high molecular weight DNA was abundant after electrophoresis on 0.4% agarose gels.

6.2.6 POLYMERASE CHAIN REACTION

Genomic DNA extracted as described above, was subjected to amplification by the polymerase chain reaction (PCR) (Saiki et al. 1988). One set of primers was designed to amplify a fragment of the chimeric *kan* gene that codes for neomycin phosphotransferase II (NPTII). The fragment extends from the *nos* promoter at the 5′ end of the gene to 150 bp beyond its 3′ termination sequences (Chee et al. 1989). The PCR product is a fragment of 1052 bp. Another set of primers was constructed to amplify PRV coat protein gene sequences. The oligomers, CTCTTTTGTGTCGTAGAATTGAGTCG and GGTGAAACAGGGTCGAGTCAG were designed to amplify a 992-bp fragment (J. Slightom, unpublished data).

Perkin-Elmer/Cetus Corporation (Norwalk, CT) PCR reagents were used exclusively. The reaction mixture consisted of the following volumes for each 0.5-ml reaction vessel: 16 μl of 2.5 mM dNTP mixture, 10 μl of PCR buffer, 10 μl of 25 mM $MgCl_2$, 1.0 μl of Taq polymerase ("Amplitaq"), and water for a total volume of 95 μl. About 400 pmoles or 0.02 to 0.03 μg of each primer was used for one reaction. The primers were added to each vessel after dispensing the reaction mixture. Genomic DNA was added last, the amount varied between 0.2 to 1.0 μg in about 5.0 μl of DNA extract.

Standard PCR conditions, as recommended by Perkin-Elmer/Cetus, were followed: one minute for denaturation at 94^{o}C, two minutes for annealing at 55^{o}C, and three minutes for extension at 72^{o}C, for 40 cycles. Ten μl of each PCR reaction product was size-separated with molecular weight markers (*Hin*dIII lambda and 1-kb ladder, Gibco/BRL, Grand Island, NY) on 0.8% agarose gels containing 1.2 μM ethidium bromide, and the gel was photographed.

6.2.7 SOUTHERN HYBRIDIZATION

Probes were prepared by large scale plasmid isolation after the protocol of Maniatis et al. (1982). Cesium chloride centrifugation was used to isolate plasmid pKS4 containing *kan* from *E. coli*. Plasmid DNA was digested with *Nco*I and *Bgl*II to release a 600-bp fragment containing *kan*

sequences (Mazodier et al. 1985). The fragment was gel-purified on 1% agarose, electroeluted (Maniatis et al. 1982), and concentrated with an Elutip-d column (Schleicher and Schuell, Keene, NH).

The coat protein gene probe was prepared from pPRV117 (Quemada et al. 1990) by digestion with *EcoRI* to release a 500-bp fragment from the 3′ end of the chimeric gene. The fragments were electroeluted and concentrated as described.

Plasmid DNA in 20-ng batches were labelled with (α-^{32}P)dCTP using a random-priming kit (Boehringer-Mannheim) after the protocol of Feinburg and Vogelstein (1983). Volumes of 0.1 and 0.2 μl of *kan* and coat protein gene DNA, respectively, were mixed with 9.9 μl water, the microfuge tubes were sealed by fusing caps to the tubes, and the mixtures were boiled for ten minutes to denature the DNA. Before quenching on ice, 2.0 μl of reaction mixture containing the random primers were added to each tube. After the quenched samples were pulsed in a microfuge, 3.0 μl of a 1:1:1 mixture of 0.2 mM dATP, dGTP, and dTTP, 5.0 μl of (α-^{32}P)dCTP (3000 Ci mM^{-1}), and 1.0 μl (2 units) of Klenow enzyme were added. The 20-μl mixtures were incubated for 30 minutes at 37^{o}C, heated to 65^{o}C for ten minutes, and quenched on ice.

The unreacted (α-^{32}P)dCTP was removed with Sephadex G-50 spin columns (Maniatis et al. 1982). Each 20-μl volume of labelled probe was pipetted onto the bed of Sephadex G-50

along with a 100-μl TE rinse. The spin columns were centrifuged at 2000 rpm for five minutes. Counts of total and trichloroacetic acid-precipitable DNA (Maniatis et al. 1982) were taken on a Beckman LS5000 TD scintillation counter to determine the percentage incorporation of ^{32}P-dCTP and to calculate the amount of probe to add to one 15-ml hybridization reaction volume.

Southern blots were prepared from agarose gel separations of PCR products or digested genomic DNA after the protocol of Maniatis et al. (1982). Genomic DNA from putative transgenic leaves was digested with excess *Hin*dIII and *Bam*HI. About 20 μg of DNA from each isolate was digested with about 20 units of each enzyme at 37^{o}C for two hours. The digests were size-fractionated on 0.8% agarose gels and blotted onto nitrocellulose ("Duralose", Stratagene, La Jolla, CA) according to the protocol of Maniatis et al. (1982). Gels, usually 11 x 14 cm, were depurinated by gently shaking each one in 200 ml of 0.25 M HCl, ten minutes for PCR gels and 30 minutes for genomic DNA gels. Gels were denatured in the same volume of 1.0 M NaCl and 0.5 M NaOH for 30 minutes with agitation. Finally, each gel was neutralized in 200 ml of 0.5 M Tris-HCl (pH 7.4) and 1.5 M NaCl for 30 minutes. Gels were placed upsidedown on 20 x SSC-soaked 3 MM blotting paper that served as wicks in reservoirs of 20 x SSC. Nitrocellulose filters that had been floated in deionized water were placed on the gels and

covered with three layers of 3 MM paper and a 2.5-cm stack of paper towels. The transfers were blotted overnight or until the paper towels were completely saturated.

Positions of the wells were marked on the blotted filters. The filters were briefly rinsed in 5 x SSC and baked in a vacuum oven at 80^{o}C for two hours. Filters were incubated in prehybridization solution (6 x SSC, 5 x Denhardt's solution, 1% SDS, and 50 μg ml^{-1} denatured and fragmented salmon sperm DNA) for at least four hours before the random-primed probes were added to the filters in prehybridization solution. The probes were boiled for ten minutes and quenched on ice prior to hybridization. Scintillation counts of the incorporated radioactivity were usually about 5 x 10^{6} cpm μl^{-1}. Filters were hybridized for at least 48 hours at 65^{o}C, rinsed briefly and washed with low stringency buffer (2.5 x SSC, 0.1% SDS) at room temperature for 15 minutes and at 65^{o}C for 15 minutes. A final high stringency wash (0.1 x SSC and 0.1% SDS) followed at 65^{o}C for 15 minutes. Wet filters were wrapped in plastic wrap, heat-sealed, and autoradiograms were made with Kodak OMAT X-ray film exposed for two to four days.

6.2.8 ELISA ASSAYS FOR PRESENCE OF COAT PROTEIN

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) technique (Clark and Adams 1977),

employing a polyclonal antibody developed by Gonsalves and Ishii (1980), was used to assay for coat protein in putative transgenic materials. About 100 mg fw of somatic embryos from putative transgenic isolates or leaves from plants expressing GUS were ground with a mortar and pestle in 5.0 ml of phosphate extraction buffer at 4°C. The buffer consisted of 0.25 M K_2HPO_4 and 0.25 M KH_2PO_4, pH 7.5, made by mixing 900 ml of 0.25 M K_2HPO_4 with 100 ml of 0.25 M KH_2PO_4 and adjusting the pH of the mixture to 7.5. For 1.0 liter of buffer, 0.1 M EDTA is added.

Prior to sample extraction, microtiter plates were coated with batch "E 11-87" fractionated gamma globulin (D. Gonsalves) that was diluted to a concentration of 1.0 $\mu g\ ml^{-1}$ with coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.20 g NaN_3, water to 1.0 liter, pH 9.6). Two hundred μl of the antibody solution was pipetted into each well. The plates were covered with Parafilm and incubated overnight at 4°C. Coated plates were washed three times with PBS-Tween (8.0 g NaCl, 200 mg KH_2PO_4. 2.9 g $Na_2HPO_4 \cdot 12 H_2O$, 200 mg KCl, water to 1.0 liter, pH 7.4, add 0.5 ml Tween 20) and blotted dry. Wells were filled with 200 μl of the crude plant extracts and incubated at 4°C overnight.

The microtiter wells were rinsed three times with PBS-Tween and filled with preadsorbed gamma globulin conjugate solutions. The preadsorption solution, used to decrease nonspecific reactions, was prepared with healthy zucchini

leaf extracts. About 5.0 g fw of leaves from the zucchini cultivar 'Ambassador' were ground in 95 ml of enzyme conjugate buffer (4.0 g PVP-40, 0.4 g nonfat dry milk, 200 ml PBS-Tween). PVP-40 was obtained from Sigma Chemical Co. A 1/2000 dilution of the gamma globulin conjugate (2.5 μl) was added to 500 μl of zucchini extract and 4.5 ml of enzyme conjugate buffer, and preadsorbed for 15 minutes at room temperature. Each well was filled with 200 μl of the preadsorbed solution. The plates were incubated overnight at 4^{o}C.

Conjugate-treated wells were rinsed three times with PBS-Tween and 220 μl per well of substrate solution was added. The alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma #104-105, Sigma Chemical Co.), was dissolved in substrate buffer (97 ml diethanolamine, 1.0 liter water, pH 9.8) at a concentration of 1.0 $\mu g\ \mu l^{-1}$. After incubating for one hour at room temperature, 30 μl of 3.0 M NaOH was added to each well, and optical density at A_{405} was determined with a Titertek plate reader.

Positive controls consisted of extracts from 100 mg fw of PRV-infected leaves, and negative controls were extracts from 100 mg fw of somatic embryos and leaf tissues of healthy, untransformed papayas.

6.3 RESULTS AND DISCUSSION

6.3.1 SELECTION BY KANAMYCIN RESISTANCE

All putative transgenic embryos (Table 6.1) were isolated on media containing 257 μM kanamycin. Bacterial contamination did not appear to affect the yield of transgenic isolates since about 50% of the total number of bombarded plates appeared to contain bacterial contaminants and were treated with additional antibiotics shortly after particle bombardment (Chapter 5). An equal number of kanamycin-resistant somatic embryos were recovered from plates treated for contamination and from uncontaminated plates (Table 6.1). The close correlation of construct PRV-19-5 with contamination was apparently due to bombardment of one or several contaminated zygotic embryo cultures. Apparently, the contamination was spread to most of the other cultures bombarded during the same session. Each construct was delivered in different bombardment sessions.

Isolates on selective medium were recovered as early as four months and as late as 23 months after particle bombardment. In Table 6.1, names prefaced by the same number, for example, "39", designate different transgenic isolates recovered from the same plate. Since zygotic embryo isolates, for example, 39-1 and 39-3, were recovered from different zygotic embryos, they represent different

transformation events. Some of the somatic embryo isolates may be identical because they were recovered from the same large tissue clusters at different times. Nevertheless, the isolates were given different names. The isolates 27-3 to 27-7 may have developed from a single transformed embryo that was GUS-negative, while GUS-positive isolates 27-1 and 27-2 may represent a different transformation event on that same culture plate.

Some isolates regenerated plants that continued to grow vigorously in the presence of kanamycin. Other isolates grew slowly in the presence of kanamycin and were regenerated without the selective agent. PCR amplification of DNA extracted from leaves of all regenerated plants showed the 1-kb sequence for *kan* (Fig. 6.1A, 6.3), regardless of whether kanamycin was present in the regeneration medium. Apparently, initial selective growth of somatic embryos on kanamycin-containing medium was sufficient for the isolation of plants transgenic for *kan*. Southern hybridizations of PCR blots with the *kan* probe confirmed the presence of *kan* gene in regenerated plants (Fig. 6.1B).

6.3.2 SUITABLE TARGET TISSUES

Freshly explanted hypocotyl sections were not suitable targets for transformation with the particle gun

(Table 6.2). A single GUS-positive embryogenic callus was produced from one hypocotyl section bombarded with the pGA482GG/cpPRV-4 construct. It grew to 1-cm in diameter on medium containing 129 μM kanamycin, but stopped growing on 257 μM kanamycin. None of the ten remaining plates containing hypocotyl sections produced kanamycin-resistant sectors during the year following bombardment, and the plates were discarded. The concentration of the selection agent may have been too high to permit survival and growth (Klee et al. 1987).

The embryogenic callus cultures were the simplest to prepare for bombardment. Seventeen percent of the plates of bombarded embryogenic calli gave rise to a total of 20 kanamycin-resistant isolates over a two-year culture period (Table 6.2). The efficiency on a fresh weight basis for callus/embryo clusters was around 1.14 isolates/g fw of bombarded tissues (Table 6.2). Regeneration of plants from the embryogenic calli was fairly difficult. Many of the isolates developed aberrant structures rather than shoots, even after removal from the selective agent. Only four isolates regenerated, and one of these was abnormal. Shoots from the fourth line, 29-1, resembled virus-infected or herbicide-damaged plants with elongated, broom-shaped leaves. The shoots rooted, but leaf development still appeared abnormal. Six months of culture on media containing 2,4-D prior to bombardment may have affected

Table 6.1 Characterization of transgenic isolates from particle bombardment

Zygotic embryo cultures

Isolate No.	SE GUS	Leaf GUS	PCR NPT	PCR CP	SH NPT	SH CP	CV	PRV	CULT CONT
37-1	-	-	?	?	?	?	K	4	-
39-1	+	+	+	+	+	+	K	4	-
39-3	-	?	?	?	?	?	K	4	-
40-1	-	-	nd	nd	nd	nd	K	19-5	+
41-1	+	+	+	+	?	?	K	4	-
44-1	+	+	+	-	?	?	K	19-5	+
46-1	-	-	?	?	?	?	SS	19-5	+
47-1	-	nd	nd	nd	nd	nd	SS	4	-
49-1	-	nd	nd	nd	nd	nd	SS	4	+
49-2	+	+	?	?	?	?	SS	4	+
50-1	-	-	nd	nd	nd	nd	SS	19-5	+
50-2	-	-	?	?	?	?	SS	19-5	+
50-3	-	-	?	?	?	?	SS	19-5	+
54-1	-	-	+	-	?	?	SS	19-5	+
55-1	+	+	+	+	+	+	SS	4	-
57-1	-	-	?	?	?	?	SS	4	-
59-1	+	+	+	+	+	+	SS	4	-
59-2	-	-	?	?	?	?	SS	4	-
60-1	+	+	?	?	?	?	SS	19-5	+
60-2	-	-	?	?	?	?	SS	19-5	+
60-3	+	+	+	+	?	?	SS	19-5	+
62-1	-	-	+	-	+	-	SS	19-5	+
62-2	-	-	+	-	+	-	SS	19-5	+
62-3	-	nd	nd	nd	nd	nd	SS	19-5	+
62-5	-	-	+	-	?	?	SS	19-5	+
63-1	-	-	?	?	?	?	SS	4	-
64-1	-	-	?	?	?	?	SS	19-5	+
64-2	-	?	?	?	?	?	SS	19-5	+

Somatic embryo cultures

Isolate No.	SE GUS	Leaf GUS	PCR NPT	PCR CP	SH NPT	SH CP	CV	PRV	CULT CONT
19-1	+	+	+	+	?	?	K	4	-
25-1	-	-	nd	nd	nd	nd	K	4	-
27-1	+	?	?	?	?	?	K	4	-
27-2	+	?	?	?	?	?	K	4	-
27-3	-	?	?	?	?	?	K	4	-
27-4	-	?	?	?	?	?	K	4	-
27-5	-	?	?	?	?	?	K	4	-
27-6	-	?	?	?	?	?	K	4	-
27-7	-	?	?	?	?	?	K	4	-

Table 6.1. (Continued) Characterization of transgenic isolates from particle bombardment

Isolate No.	SE GUS	Leaf GUS	PCR NPT	PCR CP	SH NPT	SH CP	CV	PRV	CULT CONT
29-1	+	+	+	+	?	?	K	4	-
29-2	+	?	?	?	?	?	K	4	-
29-3	+	?	?	?	?	?	K	4	-
30-1	-	?	?	?	?	?	K	19-5	+
30-2	-	?	?	?	?	?	K	19-5	+
30-3	-	?	?	?	?	?	K	19-5	+
30-4	-	?	?	?	?	?	K	19-5	+
30-5	-	?	?	?	?	?	K	19-5	+
30-6	-	?	?	?	?	?	K	19-5	+
33-1	-	nd	nd	nd	nd	nd	SS	4	-
33-2	-	-	?	?	?	?	SS	4	-

SE GUS = histochemical assay for GUS expression in somatic embryos
Leaf GUS = histochemical assay for GUS expression in leaves
PCR NPT = amplification of NPTII sequences in leaves
PCR CP = amplification of coat protein sequences in leaves
SH NPT = Southern hybridization with NPTII probe
SH CP = Southern hybridization with coat protein probe
CV = cultivar
PRV = type of PRV construct, pGA492GG/cpPRV-4 or -19-5
CULT CONT = culture contamination following bombardment
K = 'Kapoho'
SS = 'Sunset'
nd = not determined, isolate line lost

regeneration. However, nontransformed cultures that had been exposed to 2,4-D for at least six months regenerated normal-looking plants. Thus, transformation alone could have disrupted genes important for normal development.

The immature zygotic embryos were the most labor-intensive targets to prepare, but they yielded the largest numbers of transgenic embryos and plants (Table 6.2). Seventy-five percent of the plates of bombarded zygotic embryos yielded at least one transgenic embryo isolate (Table 6.2). With about 100 zygotic embryos per target plate, the transformation efficiency was about 1.38% (Table 6.2). It is assumed that an isolate represents a single transformation event; however, it is possible that one zygotic embryo could produce more than one transgenic line. Seventy percent of the zygotic embryo isolates regenerated normal-looking plants (Table 6.2, Fig. 6.9), the other 30% were either lost (8) or are still being regenerated (2). Seven isolates (19-1, 39-1, 55-1, 59-1, 60-3, 62-1, and 62-2) have been micropropagated and are growing under greenhouse conditions.

6.3.3 GUS EXPRESSION AND THE PRESENCE OF COAT PROTEIN SEQUENCES

About one third (ten out of 27, Table 6.1) of the regenerated shoot isolates were GUS-positive when young

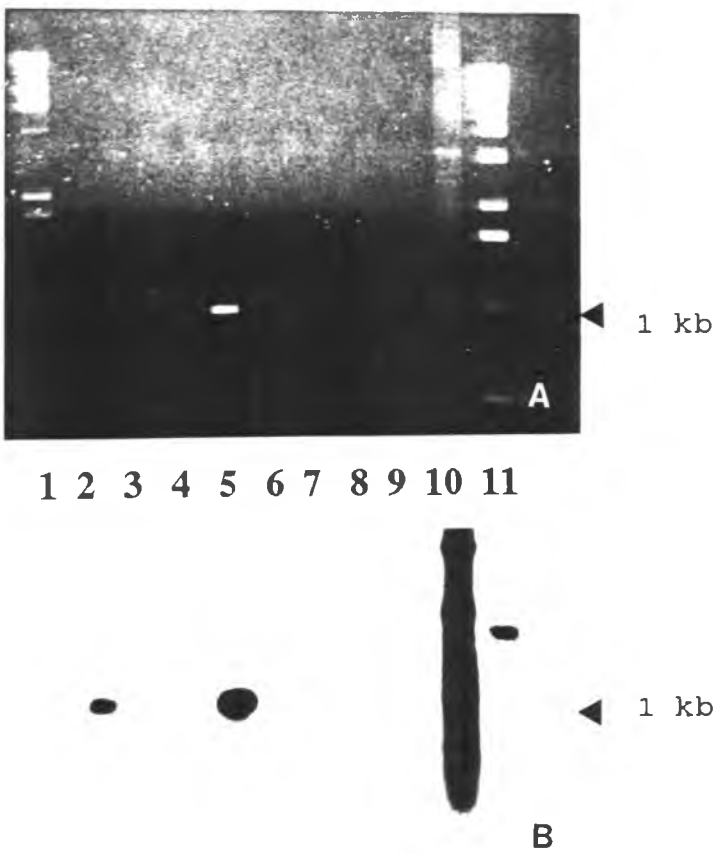

Fig. 6.1. PCR-amplified genomic DNA from untransformed and transgenic papaya leaves using *kan* primers. A) Ethidium bromide-stained PCR products. Lane 1. *Hin*dIII lambda molecular weight (MW) markers, lane 2. untransformed papaya, lane 3. 62-2 (NPTII dot blot+, GUS-), lanes 4, 6 to 9. 62-1 (NPTII dot blot+, GUS-), lane 5. 55-1 (GUS+), lane 10. pGA482GG (positive control for *kan*), lane 11. MW weight markers, Gibco/BRL 1-kb ladder. B) Southern hybridization of the gel-separated PCR products shown in Fig 6.1A. Each lane was loaded with 10% (10 μl) of a 40-cycle amplification product. The PCR products were not quantified. The nitrocellulose filters were probed with an *NCo*I/*Bgl*II-restricted 600-bp fragment from pKS4 that was labelled with ^{32}P by random priming.

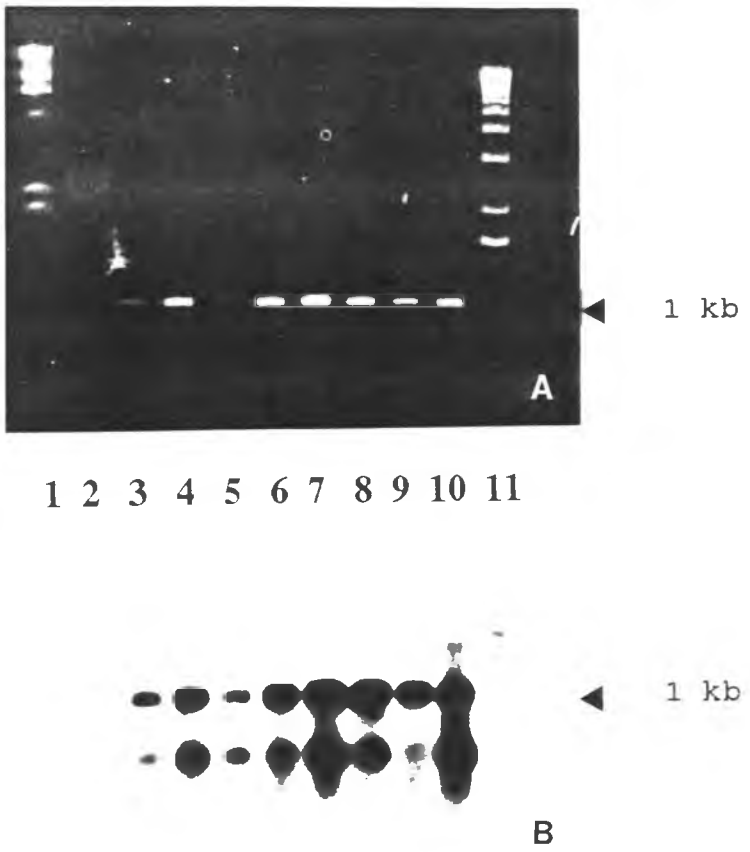

Fig 6.2. PCR-amplified genomic DNA from untransformed and transgenic papaya leaves using PRV coat protein gene primers. A) Ethidium bromide-stained PCR products. Bands in lanes 1 and 2 are believed to be contaminants from plasmid DNA isolation experiments. Lane 1. *Hin*dIII lambda MW markers, lane 2. untransformed papaya, lane 3. 62-2 (NPTII dot blot+, GUS-), lanes 4, 6 to 9. 62-1 (NPTII dot blot+, GUS-), lane 5. 55-1 (GUS+), lane 10. pPRV117 (positive control for coat protein gene), lane 11. MW weight markers, Gibco/BRL 1-kb ladder. B) Southern hybridization of the gel-separated PCR products shown in Fig 6.2A. Each lane was loaded with 10% (10 μl) of a 40-cycle amplification product. The PCR products were not quantified. The nitrocellulose filters were probed with an *Eco*RI-restricted 500-bp fragment from pPRV117 that was labelled with ^{32}P by random priming. Strong hybridization of DNA in lane 10 was probably due to large amount of pPRV117 plasmid DNA used for PCR amplification.

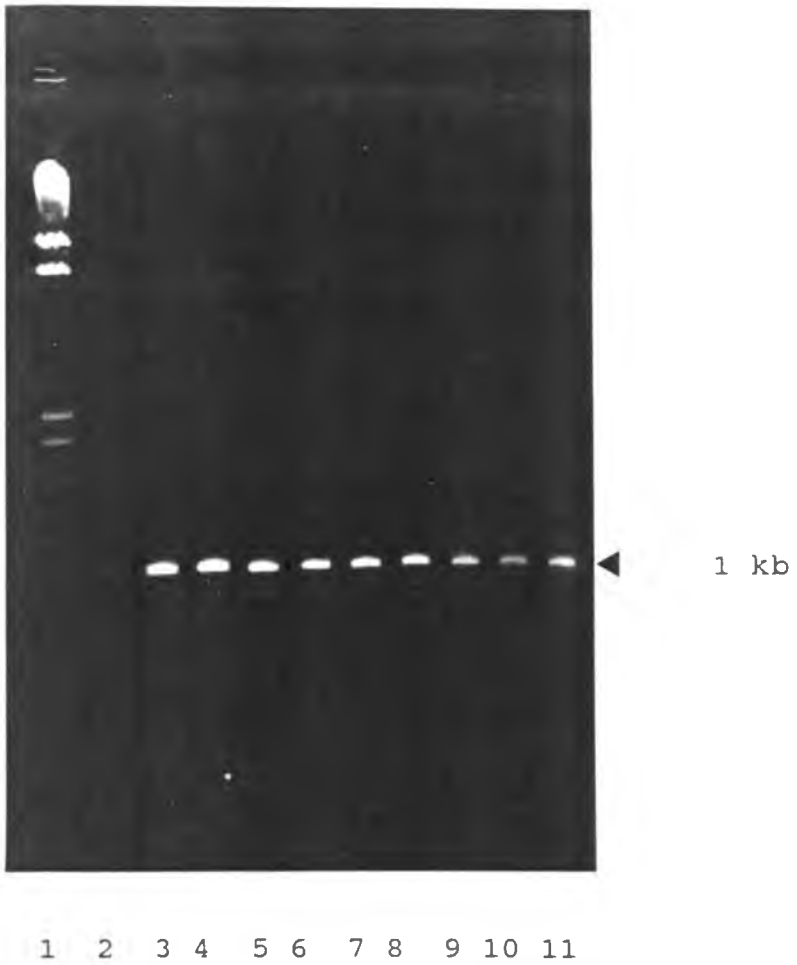

Fig. 6.3. PCR-amplified genomic DNA from untransformed and transgenic papaya leaves using *kan* primers. Lane 1. *Hin*dIII lambda MW markers, lane 2. untransformed papaya, lane 3. 55-1 (GUS+), lane 4. 19-1 (GUS+), lanes 5 and 6. 29-1 (NPTII dot blot+, GUS+), lane 7. 44-1 (GUS+), lane 8. 54-1 (GUS-), lane 9. 62-5 (GUS-), lane 10. 60-3 (GUS+), lane 11. pGA482GG (NPTII+ control). Each lane was loaded with 10% (10 μl) of a 40-cycle amplification product.

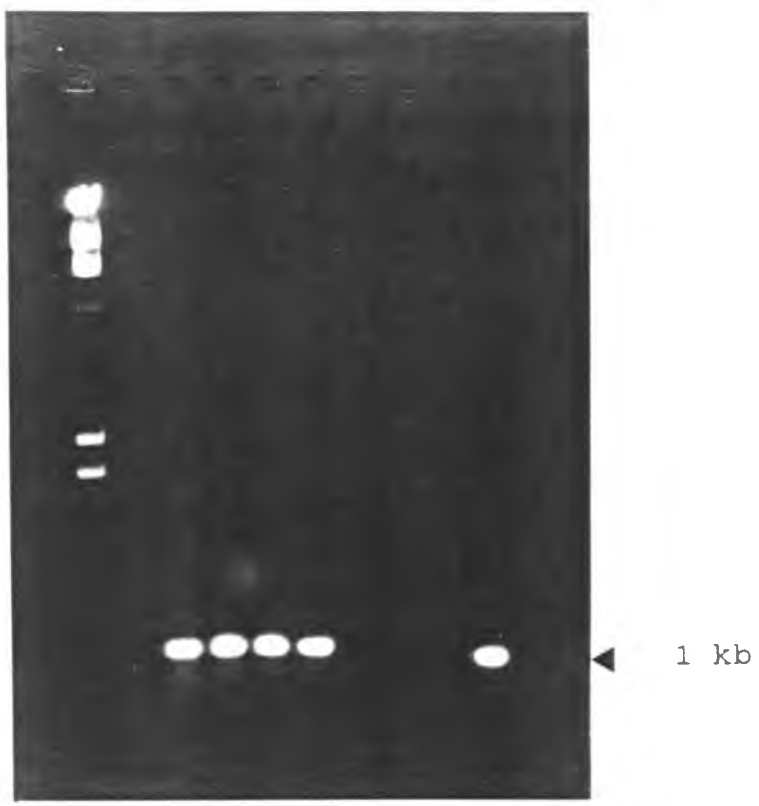

Fig. 6.4. PCR-amplified genomic DNA from untransformed and transgenic papaya leaves using PRV coat protein gene primers. Lane 1. *Hin*dIII lambda MW markers, lane 2. untransformed papaya, lane 3. 55-1 (GUS+), lane 4. 19-1 (GUS+), lanes 5 and 6. 29-1 (NPTII dot blot+, GUS+), lane 7. 44-1 (GUS+), lane 8. 54-1 (GUS-), lane 9. 62-5 (GUS-), lane 10. 60-3 (GUS+).

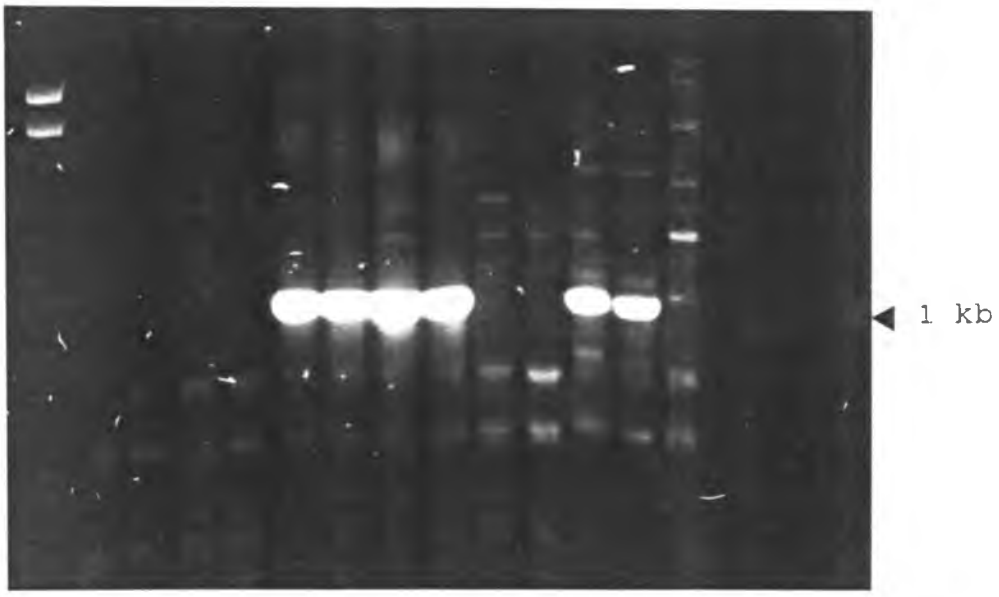

Fig. 6.5. PCR-amplified genomic DNA from untransformed and transgenic papaya leaves using PRV coat protein gene primers. Lane 1. *Hin*dIII lambda MW markers, lanes 2, 10, and 11. untransformed papaya, lane 3. 62-2 (NPTII dot blot+, GUS-), lane 4. SAI-1D-1 (GUS+) from *Agrobacterium*-mediated transformation, lane 5. 44-1 (GUS+), lane 6. 39-1 (GUS+), lanes 7, 12, and 13. 55-1 (GUS+), lanes 8 and 9. 59-1 (GUS+), lane 14. 62-1 (NPTII dot blot+, GUS-). Each lane was loaded with 10% (10 μl) of a 40-cycle amplification product. Several untransformed control samples served as contamination indicators.

Fig. 6.6. Southern hybridization of PCR-amplified DNA (coat protein gene primers) from untransformed and transgenic papaya leaves. Lane 1. untransformed papaya, lane 2. 62-1 (NPTII+, GUS-), lane 3. 62-2 (NPTII+, GUS-), lanes 4 and 5. 55-1 (NPTII+, GUS+), lane 6. 59-1 (NPTII+, GUS+), lanes 7 and 8. 39-1 (NPTII+, GUS+). Each lane was loaded with 10% (10 μl) of a 40 cycle-amplified product. The nitrocellulose filters were probed with an *EcoR*I-restricted 500-bp ^{32}P-labelled fragment of cpPRV from pPRV117. The bands in lanes 1 and 2 are believed to be due to plasmid contamination.

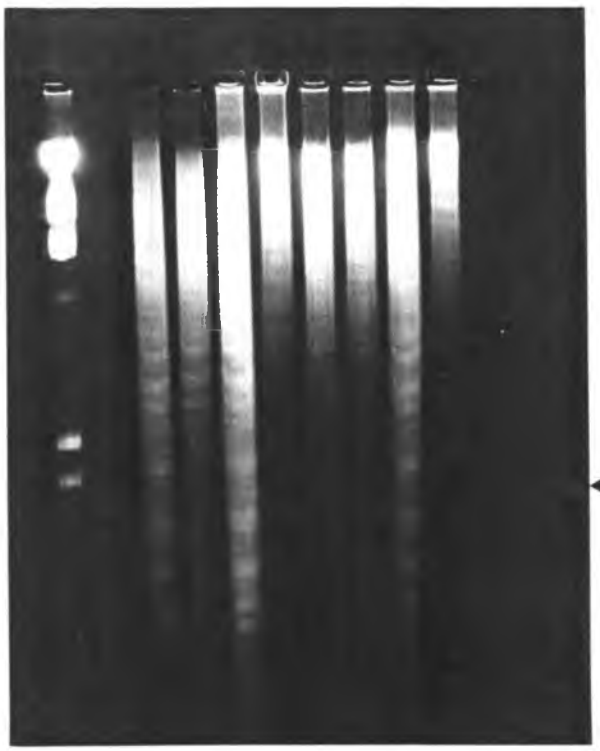

Fig. 6.7. Genomic DNA from untransformed and transgenic papaya leaves. Ethidium bromide-stained *Hin*dIII and *Bam*HI restriction digests. Lane -1. *Hin*dIII lambda MW markers, lane 0. space, lane 1. untransformed papaya, lane 2. 62-1 (NPTII+, GUS-), lane 3. 62-2 (NPTII+, GUS-), lanes 4 and 5. 55-1 (NPTII+, GUS+), lane 6. 59-1 (NPTII+, GUS+), lanes 7 and 8. 39-1 (NPTII+, GUS+). About 20 μg per lane was loaded and the DNA was electrophoresced on 0.8% agarose gels containing ethidium bromide.

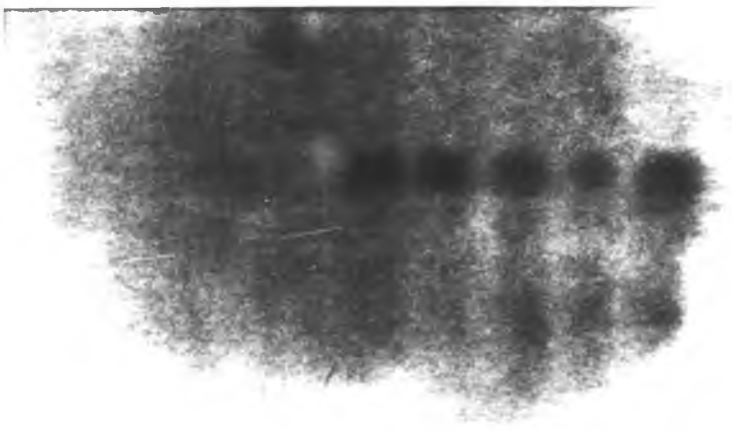

Fig. 6.8. Southern hybridization of restriction digests shown in Fig. 6.7. Lane 1. untransformed papaya, lane 2. 62-1 (NPTII+, GUS-), lane 3. 62-2 (NPTII+, GUS-), lanes 4 and 5. 55-1 (NPTII+, GUS+), lane 6. 59-1 (NPTII+, GUS+), lanes 7 and 8. 39-1 (NPTII+, GUS+). The gel shown in Fig. 6.5A was blotted onto nitrocellulose and probed with an *Eco*RI-restricted 500-bp ^{32}P-labelled fragment of cpPRV from pPRV117. The hybridizing fragments are 1.7 kb in size (arrow).

Table 6.2. Efficiency of particle gun transformation

Amount of tissue bombarded	No. of positive plates/ total	No. of iso- lates	Effi- ciency	No. of plants
~2000 hyp. sections	1/11	1	0.05%	0
~17.5 g fw hyp. calli	6/35	20	1.14/g	4
~2400 zyg. embryos	18/24	33	1.38%	23

leaves were tested using the histochemical assay. Leaves that did not express GUS also did not show PCR amplification of coat protein sequences in extracted DNA (Figs. 6.2, 6.4, and 6.5). Six GUS-positive isolates (19-1, 29-1, 39-1, 55-1, 59-1, 60-3) also showed coat protein sequence amplification with PCR (Figs. 6.2, 6.4, 6.5). One isolate that was strongly positive for GUS (44-1) and *kan* (Fig. 6.3) was negative for CP sequences (Figs. 6.4 and 6.5).

Three isolates (39-1, 55-1, and 59-1) that were subjected to genomic Southern hybridization showed evidence for coat protein gene sequences (Fig. 6.8). Two isolates (62-1 and 62-2) that were GUS-negative showed neither coat protein sequence amplification with PCR (Figs. 6.2A and 6.5) nor hybridization with coat protein probes in Southern blots of PCR (Figs. 6.2B and 6.4). Figures 6.2 and 6.4 show contamination of negative control, 62-2, and 62-1 (GUS-negative), respectively, with amplified coat protein sequences. This contamination was attributed to use of the same centrifuge tubes for both plasmid isolation and genomic DNA isolation without special attention to washing of the tubes between experiments. Acid-washing of tubes in subsequent experiments eliminated the contamination problem. Southern blots of the genomic digests of DNA (Fig. 6.8) from the samples amplified in Fig. 6.6 confirmed the absence of coat protein sequences in the negative control and 62-1. In Fig. 6.5, DNA in lane 4 was extracted from leaves of papayas

transformed with *Agrobacterium tumefaciens* carrying only genes for kanamycin resistance and GUS. This sample served as another negative control for coat protein sequences.

GUS expression varied between individual isolates and within the same individual. Cut leaves of the isolate 44-1 consistently responded with dark blue staining at all injured surfaces. On the other hand, 41-1, that stained intensely blue as somatic embryos (Fig. 5.2F), was initially labelled GUS-negative when fully expanded leaves were assayed. Only the youngest leaves, about 1/3 to 1/2 the size of fully expanded leaves, turned pale blue with X-gluc. Leaves from different isolates differed in the pattern of expression. Some stained most intensely in the vascular tissues of the leaves and in the petiole (59-1), while others stained in circular spots or wedge sectors on the lamina of young leaves, and older leaves did not stain (60-3). Unlike somatic embryos that can produce false-positive light blue spots after long exposure (more than 12 hours) to X-gluc, leaves stained faster (ten minutes to one hour), and never exhibited the false-positive blue response. GUS expression was not uniform between transgenic isolates nor within an individual as observed by Benfey and Chua (1989). Some of the isolates may be chimeric, but the apparent developmental regulation of GUS expression in isolates like 41-1 and 60-3 make it difficult to determine if lack of expression is due to difference in tissue genotype or

developmental regulation. Lateral branches from three different somatic embryos (55-1-1, 55-1-2, and 55-1-5) of isolate 55-1 have been propagated and assayed at random for GUS expression. GUS expression was always positive. As larger numbers of individual somatic embryos from each isolate are assayed for gene insertion and expression, evidence of chimeras may be found.

The correlation of GUS expression with PCR amplification of the coat protein gene makes the GUS reporter gene a good indicator of the presence of coat protein sequences. The absence of PCR-amplification of coat protein gene sequences in samples negative for GUS expression suggest that genes can be lost during transformation with the particle gun. The GUS-positive and *kan*-positive isolate, 44-1 (Table 6.1) is interesting because it appears to have received genes for these two enzymes while the CP gene located between the two in the construct was lost. Genetic rearrangement resulting in loss of genes has been documented in transformation with *Agrobacterium*, direct DNA uptake, and the particle gun (Klee et al. 1987, Klein et al. 1988b).

Fig. 6.9. Transgenic papaya plants that express GUS and that show evidence for the coat protein gene of PRV with PCR and Southern hybridizations. Left, 55-1, right, 39-1. Scale = 2 cm.

6.3.4 ELISA ASSAY

The expression of viral coat protein was assayed using ELISA. Coat protein could not be detected in the transgenic samples, although the PRV-infected control was strongly positive with an optical density average value and standard error at A_{405} of 2.84 ± 0.054 (n = 6). This average was 13.2 times that of the untransformed negative control (0.215 ± 0.009, n = 6). The optical density averages (n = 6) from the coat protein gene-positive and negative isolates, 55-1-2 and 62-1-1, respectively, were both 1.39 times greater than the negative control. In addition, averages from coat protein gene-positive samples 29-1, 39-1-1, and 39-1-2 (n = 3) were 1.64, 1.01, and 1.02 times greater, respectively, than the negative control, while the optical density average from another coat protein gene-negative sample, 62-2-1 (n = 3), was 1.22 times greater than the negative control. Since optical density averages from the coat protein gene-positive samples were equivalent to the samples showing no integration of the gene, ELISA is apparently not sensitive enough to detect the protein. Estimates of coat protein content in transgenic plants produced by other investigators range from about 0.001 to 0.8% of total soluble protein (Cuozzo et al. 1988, Tumer et al. 1987). On the other hand, coat protein may not be transcribed and/or translated in some samples. The negative ELISA response may make it

possible to assay for the presence of replicating viruses in transgenic plants after inoculation with PRV, although this might be achieved less ambiguously by use of antibodies specific for the nuclear inclusion protein of PRV (Yeh and Gonsalves 1984b).

6.3.5 MICROPROPAGATION AND ROOTING

Cuttings were removed from unrooted propagules grown in MPII liquid medium or in MS/IBA agar medium or from rooted plants in grown in MSV medium. Cuttings rooted in MS/IBA agar medium with no significant difference at the 1% level in the number of days for root formation on shoots from several transgenic isolates. The F test showed no significant difference between cuttings from five isolate lines grown on MPII micropropagation medium prior to rooting on MS/IBA (Table 6.3). In addition, significant differences were not found with the t test at the 1% level between pairs of cuttings of the same isolate grown on different media prior to root initiation.

The MPII medium was preferable for multiple shoot proliferation because strong apical dominance in the rooted cuttings in MSV or unrooted cuttings in MS/IBA induced single shoot development only. Two to ten shoots were removed from propagules grown in an equal volume of MPII liquid medium. Between 47 and 100% of the cuttings removed

Table 6.3. Number of days for rooting of micropropagated transgenic papaya shoots. Cuttings were removed from propagules grown on various media. Analysis of variance tables summarize the data for the isolate lines

Isolate No.	Medium prior to cutting	No. of days for rooting	% Rooted after two months
19-1	MSV	23.0	47
55-1	MSV	22.6	50
60-3	MSV	13.4	54

Source	df	SS	MS	F	F0.05	F0.01
Total	23	4531.33				
Isolate	2	475.58	237.79	1.18	3.74	6.51
Rep.	7	1242.67	177.52	0.88	2.76	4.28
Error	14	2813.08	200.93			

Isolate No.	Medium prior to cutting	No. of days for rooting	% Rooted after two months
39-1	MPII	27.0	67
44-1	MPII	24.3	69
54-1	MPII	19.3	100
59-1	MPII	16.4	89
62-5	MPII	21.9	100

Source	df	SS	MS	F	F0.05	F0.01
Total	39	6065.5				
Isolate	4	551.75	137.94	0.97	2.71	4.07
Rep.	7	1528.70	218.39	1.53	2.36	3.36
Error	28	3985.05	142.32			

Isolate No.	Medium prior to cutting	No. of days for rooting	% Rooted after two months	t Value
54-1	MPII	19.3	100	0.45
54-1	MS/IBA	17.5	90	
55-1	MSV	22.6	50	0.00
55-1	MS/IBA	22.4	67	
19-1	MSV	23.0	47	0.30
19-1	MPII	19.9	67	
54-1	MS/IBA	17.5	90	0.73
55-1	MS/IBA	22.4	67	

from plants grown in MSV or shoots grown in MPII or MS/IBA rooted after a total of two months on MS/IBA medium (Table 6.3).

Transgenic papaya plants expressing NPTII (Chapter 5) and GUS have been regenerated following particle bombardment of embryogenic tissues and selection on media containing kanamycin. Genomic DNA showed evidence for the presence of sequences of the chimeric genes for PRV coat protein and NPTII. The highest efficiency of transformation and plant regeneration was obtained with immature zygotic embryos that were pretreated with 2,4-D, while freshly explanted hypocotyl sections did not yield any transgenic plants. Transgenic plants are being grown for seedling progenies for segregation analysis and testing of virus resistance.

A preliminary experiment to test the effectiveness of coat protein-mediated virus resistance indicated that protection was conferred. Transgenic lines 62-1 (GUS-negative, CP-negative) and 55-1 (GUS-positive, CP-positive, Fig. 6.9), two tissue-cultured, nontranformed lines, and one group of micropropagated seedlings were challenge-inoculated with the virulent HA-5 strain of PRV (Yeh and Gonsalves 1984a). Virus symptoms were observed on leaves of all nontransformed plants and on 62-1, 18 days after heavy challenge inoculation while the four replicates of 55-1 were symptom-free (D. Gonsalves, unpublished data).

CHAPTER 7. *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF PAPAYA SOMATIC EMBRYOS

7.1 INTRODUCTION

Papaya is a tropical and subtropical perennial tree crop that is highly susceptible to papaya ringspot virus (PRV), a pathogen that limits production throughout the range of the crop. Modest resistance levels within this species (Conover and Litz 1978) and a relatively long generation time of 12 months (Storey 1953) make the improvement of resistance by conventional means time-consuming and uncertain. Interspecific hybridization for the purpose of moving resistance genes from related *Carica* species into commercial cultivars has been attempted (Manshardt and Wenslaff 1989a, 1989b), and interspecific hybrids have been produced that exhibit resistance to PRV under field conditions (R. Manshardt, unpublished data). However, hybrid infertility has slowed the rate of breeding progress by this approach. Genetic engineering techniques could enhance breeding programs by ameliorating disease problems more quickly (Grumet 1990).

Transformation protocols have been developed to insert virus resistance genes into commercial papaya cultivars. The coat protein gene of PRV may confer resistance to the virus, based on the strategy of Powell Abel et al. (1986),

who demonstrated genetically engineered resistance to tobacco mosaic virus (TMV) in tobacco. Tobacco plants transformed with the coat protein gene of TMV showed delayed virus symptom development when challenged with a virulent strain of TMV.

Many dicotyledonous plants are routinely transformed by co-cultivating leaf disks with disarmed *Agrobacterium tumefaciens* strains (Horsch et al. 1985). In instances where regeneration from leaf disks has proven difficult, other regeneration methods have been adapted for transformation with *Agrobacterium*. Transgenic plants expressing foreign genes for ß-glucuronidase (GUS) and neomycin phosphotransferase (NPTII), have been regenerated from soybean cotyledons attached to axillary buds (Hinchee et al. 1988), maize (Gould et al. 1991) and sunflower shoot apices (Schrammeijer et al. 1990), and walnut somatic embryos (McGranahan et al. 1988) following co-cultivation of these tissues with *A. tumefaciens*.

Pang and Sanford (1988) were able to transform papaya leaf disks with *A. tumefaciens*, but they could not regenerate plants from the leaf disk cultures. In this study, an efficient plant regeneration system was developed for papaya tissues before transformation was attempted. The tissue culture results of De Bruijne et al. (1974), Yie and Liaw (1977), Arora and Singh (1978a), and Litz et al. (1983), who demonstrated either somatic embryogenesis or

organogenesis in papaya cultures, could not be reproduced. By modifying the ovule culture method that Litz and Conover (1981a, 1982, 1983) used for high frequency embryogenesis in interspecific hybrids of papaya, a protocol for efficient embryogenesis from immature zygotic embryos of papaya was developed (Chapter 3). That work, augmented with a procedure for producing embryogenic hypocotyl calli (Chapter 4), was coupled with the protocol of McGranahan et al. (1988) to obtain transgenic papaya plants by co-cultivating embryogenic tissues with *A. tumefaciens*.

Stable transformation of papaya with the particle gun (Chapter 5) has previously been reported. Transgenic papaya somatic embryos and leaves of shoots expressed NPTII in the dot blot assay (McDonnell et al. 1987), and GUS was detected in somatic embryos with the histochemical assay (Jefferson 1987). This report documents the transformation of papaya with genes from *A. tumefaciens* cosmid vectors identical to those that were delivered into papaya with the particle gun.

7.2 MATERIALS AND METHODS

7.2.1 PLANT MATERIAL AND CULTURE CONDITIONS

Seeds of self-pollinated Hawaiian papaya cultivar, 'Kapoho', were germinated aseptically for initiation of somatic embryo cultures from hypocotyl sections (Chapter 4).

Six- to eight-month-old cultures derived from immature zygotic embryos of 'Kapoho' and 'Sunset' provided a second source of somatic embryos (Chapter 3) for *A. tumefaciens* co-cultivation.

Hypocotyls from ten-day-old seedlings were sliced into 2- to 3-mm sections and either directly co-cultivated with *A. tumefaciens* or plated on induction medium (Chapter 4) to initiate embryogenic calli. Between 20 to 40 2-mm-wide sections were plated from each seedling, depending on the length of the hypocotyl.

Embryogenic calli that developed from hypocotyl sections cultured on induction medium were subcultured on induction medium containing 2.3 to 113.1 μM 2,4-D or were subcultured to maturation and MS media to initiate somatic embryo development. Cultures were maintained for six to eight months before co-cultivation with *A. tumefaciens*.

Somatic embryo cultures, initiated from 90- to 105-day-old immature zygotic embryos, were also co-cultivated with *A. tumefaciens*. Embryos had been dissected from ovular tissues and maintained on induction media containing 4.5 to 113.1 μM 2,4-D (Chapter 3) for seven months prior to co-cultivation.

7.2.2 *AGROBACTERIUM* CULTURES

Agrobacterium tumefaciens strains C58-Z707 (Hepburn et al. 1985) and A208.35 (Hinchee et al. 1988) were used to transfer binary cosmid vectors derived from pGA482 (An 1986) into papaya. The plasmids pGA482GG (17 kb), pGA482GG/cpPRV-4 (18.6 kb), and pGA482GG/cpPRV-19-5 (18.6 kb) contained a bacterial *gus*A gene (Jefferson et al. 1986), in addition to the *kan* gene of pGA482 (An 1986). Two of the plasmids, pGA482GG/cpPRV-4 and pGA482GG/cpPRV-19-5, were engineered (H. Quemada and J. Slightom, unpublished data) with chimeric genes for the coat protein of the mild mutant strain HA 5-1 of papaya ringspot virus (PRV) (Yeh and Gonsalves 1984a).

The *A. tumefaciens* biovars C58 and A208.35 were transformed as described by Hepburn et al. (1985) and prepared for co-cultivation by the method of Hookyaas (1988). Cultures of bacteria were grown overnight in 50 ml of Luria broth containing 42.9 μM kanamycin sulfate and 25 mg l^{-1} gentamicin sulfate (Sigma Chemical Co., St. Louis, MO). The turbid, overnight cultures of *A. tumefaciens* were induced for four hours with acetosyringone (Calbiochem, San Diego, CA) prior to co-cultivation (Bolton et al. 1988). Acetosyringone was dissolved in DMSO at a concentration 0.5 M, and it was added to control Luria broth media and to the *A. tumefaciens* cultures to a produce a final concentration of 50 μM.

7.2.3 *AGROBACTERIUM* CO-CULTIVATION

7.2.3.1 HYPOCOTYL SECTIONS

Freshly sliced hypocotyl sections were immediately placed into 18 ml of liquid callus induction medium to which 2.0 ml of an induced or noninduced *A. tumefaciens* culture had been added. Control hypocotyl sections were dipped into 20 ml of medium with or without inducer. Another set of control sections was grown on plates of induction medium with no further treatment. Sections to be transformed were incubated for 30 minutes with *A. tumefaciens* to allow for bacterial attachment and subsequently were blotted dry on sterile filter papers. Each treatment contained hypocotyl sections from three seedlings. Co-cultivated and control sections were incubated on induction medium for two days at 27^{o}C in the dark.

To kill the bacteria after co-cultivation, the hypocotyl sections were shaken at 27^{o}C overnight in 20 ml of liquid callus induction medium containing 1.1 mM cefotaxime (Calbiochem). The antibiotic-containing solutions were decanted, and the hypocotyl sections were transferred to callus induction agar medium containing 0.4 mM cefotaxime. The dishes were sealed with Parafilm and stored in the dark.

In four to six weeks, half of the hypocotyl sections in each treatment were transferred to maturation medium and the

other half to induction medium. Within each medium treatment, half of the hypocotyls were placed on medium that contained 129 μM kanamycin and the other half were not exposed to the selection agent. All media contained 0.4 mM cefotaxime. Hypocotyl sections were grown in subdued light, less than 10 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), and monitored monthly for selective growth.

Bacteria grew in some cefotaxime-containing cultures, and thus a more stringent antibiotic treatment was necessary. In some cases 1.2 mM carbenicillin (Sigma Chemical Co.) was added to the 0.4 mM cefotaxime in induction medium, while in others the hypocotyl sections were treated with 61 μM rifampicin (Sigma Chemical Co.) in liquid induction medium (Haldeman et al. 1987). Cultures containing bacteria with apparent resistance to antibiotics were discarded. Once bacterial growth was under control, as determined by visual observation, the hypocotyl sections were not subcultured for five months. Contamination was monitored by streaking hypocotyls on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI).

7.2.3.2 SOMATIC EMBRYOS AND EMBRYOGENIC CALLI

In addition to freshly explanted hypocotyl sections, somatic embryos and embryogenic calli were co-cultivated with induced or noninduced *A. tumefaciens* in liquid

induction medium. Negative controls consisted of suspensions of embryogenic calli mock inoculated with Luria broth with or without the addition of acetosyringone. Approximately 500 mg of embryogenic tissues were suspended in 10 ml of induction medium. Each treatment, consisting of one to five flasks of suspended tissues, was co-cultivated with 50 µl of *A. tumefaciens* for two days at 27°C. Following the co-cultivation period, suspensions were decanted and rinsed twice with one or two volumes of induction medium. Viscous cultures were diluted with two volumes of induction medium, decanted, and rinsed. The rinsed tissues were transferred to 10 ml induction medium containing 1.1 mM cefotaxime and were shaken overnight at 27°C.

Subsequent co-cultivation experiments with embryogenic tissues were modified slightly. One-gram batches of tissues, including two-month-old suspension cultures from MS medium containing 9.0 µM 2,4-D (MS2), were suspended for a day in 20 ml of MS2 containing 2.0 ml of an induced overnight culture of *A. tumefaciens*. Following co-cultivation, the tissues were separated from the viscous bacterial suspensions with a slotted spoon, blotted on sterile paper towels, and suspended in MS2 containing 1.2 mM carbenicillin and 0.4 mM cefotaxime. After two days, half of the tissues were plated on induction medium and the other half on maturation medium, both containing 1.2 mM

carbenicillin, 0.4 mM cefotaxime, and 257 μM kanamycin. The tissues were subcultured monthly.

Re-growth of bacteria on embryogenic cultures was treated by increasing the cefotaxime concentration to 0.8 or 1.0 mM. Later, calli were suspended in induction medium containing 61 μM rifampicin for two days and then plated on induction medium containing 61 μM rifampicin. In a third treatment, the medium contained 1.2 mM carbenicillin and 0.4 mM cefotaxime. Cultures that appeared to harbor antibiotic-resistant bacteria that grew despite the three treatments were discarded.

Three weeks following the initial co-cultivation experiment, most of the uncontaminated cultures were transferred to induction medium containing 129 or 515 μM kanamycin. Growth of some cultures did not appear to be inhibited by the presence of kanamycin, and in these the concentration of the selective agent was increased from 129 to 257 μM or from 515 to 772 μM. These cultures were not subcultured for four months.

The somatic embryos that grew selectively on kanamycin were removed to maturation medium containing 257 μM kanamycin. The isolates were grown until the somatic embryos appeared mature. Mature somatic embryos were subcultured to fresh maturation medium containing kanamycin. If growth appeared to be too slow, the somatic embryos were

transferred to MS medium where germination occurred in one to two months.

Germinated somatic embryos with dark green leaves and apices were grown on MS medium until roots developed, or they were micropropagated in liquid micropropagation (MPII) medium (Lee 1987). Micropropagated shoot apices about 1-cm long were rooted on MS agar medium supplemented with 4.9 μM IBA (Chapter 4). Germinated somatic embryos and rooted cuttings were grown in sterile vermiculite moistened with MS liquid medium (MSV, Chapter 4). Leaves removed for GUS assays and DNA extraction were pooled from one or several germinated somatic embryos that arose from the same isolate.

7.2.4 GUS HISTOCHEMICAL ASSAY

Leaves were assayed for GUS expression using the histochemical assay (Jefferson 1987) as modified for papaya (Chapter 5). Sliced leaves were incubated in filter-sterilized substrate at 37^{o}C, observed after 30 minutes to four hours, and left overnight for further color development. Chlorophyll that sometimes masked the blue precipitate was removed by soaking leaves in several changes of 95% ethanol. Leaves that turned blue at the injured surfaces were scored as GUS-positive, while those scored negative were re-tested at least five times.

7.2.5 POLYMERASE CHAIN REACTION

DNA was extracted from lyophilized leaves of putative transformants and untransformed controls by the method of Dellaporta et al. (1983) as modified for papaya (Chapter 6).

PCR (Saiki et al. 1988) was performed as recommended by Perkin-Elmer/Cetus (Norwalk, CT) using the AmpliTaq PCR kit. Samples containing about 200 to 1000 ng of DNA were amplified in 40 cycles with primers for *kan* (Chee et al. 1989) or cpPRV gene (Chapter 6).

7.3 RESULTS AND DISCUSSION

7.3.1 FRESHLY EXPLANTED HYPOCOTYL CULTURES

Cultured hypocotyl sections differed in size and morphology depending on the treatment they received. Hypocotyl sections in the transformation control treatment, whether induced with acetosyringone or not, became swollen, and epidermis and cortex layers fell away from the central vascular bundles five days after plating on cefotaxime-containing medium. They resembled control hypocotyl cultures that had not been subjected to mock *A. tumefaciens* co-cultivation and that had remained on callus induction medium since culture initiation. Loosening and enlargement of epidermal and cortical cells was the typical response.

The *A. tumefaciens*-treated hypocotyl sections showed little swelling and cell-loosening in comparison. Brown spots and streaks were observed on cut ends and on the epidermis of some of the co-cultivated sections. About a month after co-cultivation, a few of the hypocotyl sections showed cell enlargement and loosening, but this was delayed compared to the controls. This apparent pathogen-elicited response was peculiar to all of the *A. tumefaciens*-treated hypocotyls.

Re-growth of bacteria on hypocotyl sections occurred even though cefotaxime was included in the induction medium. The concentration of cefotaxime was increased from 0.4 to 0.8 or 1.0 μM, but some bacteria still grew. Addition of carbenicillin or substitution of rifampicin in induction media finally controlled the problem. After 12 months with intermittent subculture passages, no embryogenic calli developed from the co-cultivated hypocotyl sections. Untransformed control sections produced embryogenic calli on induction medium. Hypocotyl tissues were discarded.

7.3.2 SOMATIC EMBRYOS AND EMBRYOGENIC CALLI

Pale yellow or white embryogenic tissues became light brown after co-cultivation, and some somatic embryos developed brown spots that resembled hypersensitivity reactions on cotyledons, hypocotyls, and near the radicle tips. Growth of the somatic embryos was apparently

inhibited by 129 μM kanamycin for a month. After that period, the concentration of the selective agent was doubled because all tissues appeared to resume growth. Even tissues initially screened with 515 μM kanamycin appeared to grow; thus, those cultures were subjected to selection with 772 μM kanamycin.

Re-growth of the bacteria on clusters of embryogenic tissues occurred three weeks after the first co-cultivation experiment. Cefotaxime, rifampicin, or cefotaxime and carbenicillin controlled bacterial growth two months after co-cultivation, and cultures were not subcultured for another four to five months. A total of nine months after co-cultivation, slow-growing, light green somatic embryo clusters developed from among the brown tissues. Portions of these clusters were tested for GUS expression. Tissues incubated in X-gluc turned blue within ten minutes to one hour, and the positive response was not due to contamination by *A. tumefaciens*. The tissues had been removed from media containing carbenicillin and cefotaxime about one month prior to the assay. Bacterial re-growth did not occur in any of the subsequent subcultures to maturation medium containing kanamycin. In addition, tissues from GUS-positive cultures remained free of bacteria when they were plated on PDA.

7.3.3 CHARACTERIZATION OF TRANSGENIC PLANTS

The somatic embryos enlarged faster on MS medium than on maturation medium, although both media contained 257 μM of the selective agent. Germination occurred in one to two months and plants were transferred to MSV or micropropagation medium. Leaves of two isolates from the pGA482GG construct and of two isolates from the pGA482GG/cpPRV-4 construct were positive for GUS (Table 7.1). A total of 11 putative transgenic isolates from the pGA482GG construct, originally from 8.5 g fw of co-cultivated somatic embryos, were identified by kanamycin-resistant growth on medium containing 257 μM kanamycin. All of the eight surviving pGA482GG isolates tested so far have been GUS-positive as somatic embryos and are being regenerated. Three isolate lines were lost.

Two pGA482GG/cpPRV-4 isolates produced chlorophyll-deficient leaves when cultured in micropropagation medium. Upon transfer of cuttings from the cytokinin-containing medium to MS medium containing IBA for rooting, normal-looking green leaves were produced. Cut surfaces of the pale and green leaves turned dark blue in the histochemical assay for GUS (Fig. 7.1). Green shoots from these two isolates again produced pale green leaves when returned to micropropagation medium. The pGA482GG isolates did not show this response. The apparent inhibition of chlorophyll

Table 7.1. Putative transgenic isolates from co-cultivation of papaya somatic embryos with *Agrobacterium tumefaciens*

Plasmid construct	Tissue type	Initial FW	No. of surviving trans-formants	GUS (+)	PCR NPTII	PCR CP
Control	HYP SE	8.5 g	0	0	0	0
pGA482GG	HYP SE	8.0 g	8	8	1[a]	0[a]
pGA482GG/cpPRV	HYP SE	7.5 g	2	2	2	2
pGA482GG/cpPRV	ZE SE	5.5 g	0	0	0	0

[a]Only one isolate tested so far
pGA482GG = binary plasmid containing genes for NPTII and GUS
pGA482GG/cpPRV = same the pGA482GG but containing cpPRV-4
HYP SE = somatic embryos derived from hypocotyl calli
ZE SE = somatic embryos derived from zygotic embryos
GUS(+) = histochemical response of somatic embryos
PCR NPTII = PCR amplification of leaf DNA with *kan* primers
PCR CP = PCR amplification of leaf DNA with coat protein gene primers

Fig. 7.1. Histochemical assay for GUS on leaves from papaya plants tranformed with *Agrobacterium tumefaciens*. On the left, pale green leaves from plant transformed with pGA482GG/cpPRV-4. On the right, green leaves from plants transformed with pGA482GG construct. Scale = 1 cm.

biosynthesis, correlated with culture in the cytokinin-containing medium, is not understood at this time.

DNA was extracted from leaves of one isolate line transformed with pGA482GG and from two lines transformed with pGA482GG/cpPRV-4. The extracts were subjected to PCR amplification using primers for both *kan* and the PRV coat protein gene. The results are shown in Figs. 7.2 and 7.3. DNA from transgenic papayas derived from particle bombardment (Chapter 5) were extracted and amplified in the same experiment for comparison with the *A. tumefaciens*-mediated transformants. In Fig. 7.2, the amplified *kan* sequence from *Agrobacterium*-mediated transformation (lanes 4 and 5) is unusual in that it is about 300 bp larger than the 1-kb sequence amplified from transformants produced by particle bombardment (lanes 3, 9, and 11) and from the positive control pGA482GG DNA (lane 10). A faint 1-kb band is visible in lanes 4 and 5 but a greater quantity of product appears at about 1.3 kb, based on the ethidium bromide fluorescence. The 1.3-kb fragment apparently was produced in smaller quantity than the 1-kb product from particle gun transformation, however. The two transformants from the pGA482GG/cpPR-4 construct (lanes 6, 7, and 8) show *kan* amplification similar to the particle gun transformants. Nearly the same range of DNA samples was subjected to amplification with CP primers in Fig. 7.3 as in Fig. 7.2, but 1-kb CP sequences were amplified in only GUS-positive

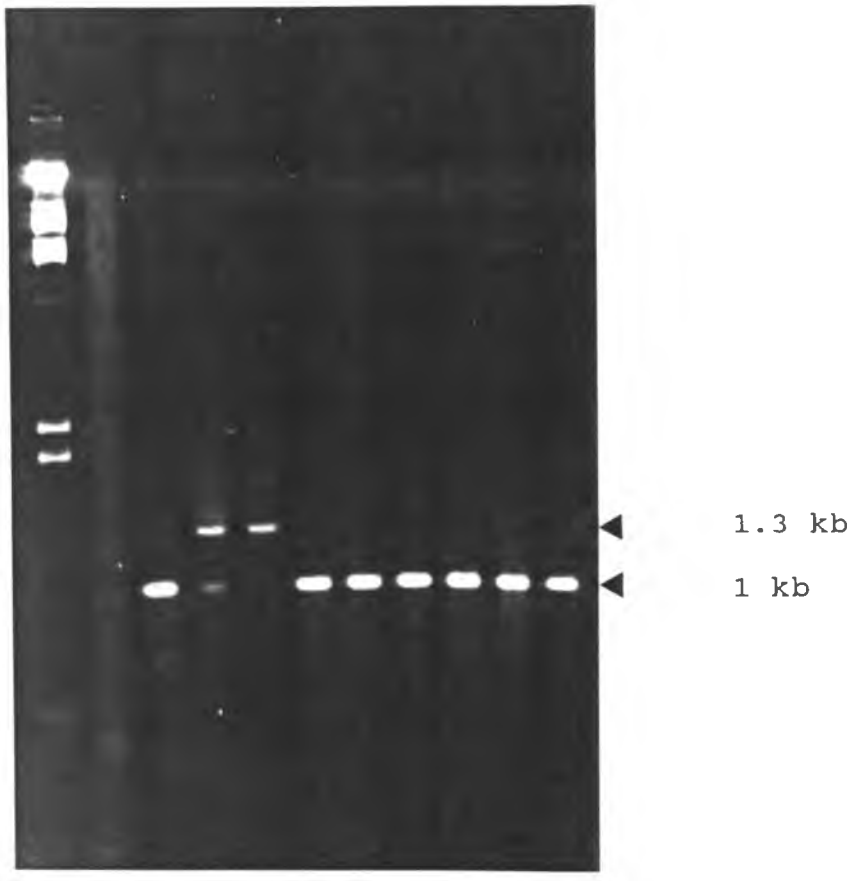

Fig. 7.2. PCR-amplified genomic DNA from untransformed and transgenic papaya leaves using *kan* primers. Lane 1. *Hin*dIII lambda MW markers, lane 2. untransformed papaya, lanes 3 and 11. 55-1 (NPTII+, GUS+, particle gun), lanes 4 and 5. *Agrobacterium* isolate SAI-1D-1 (GUS+), lane 6. *Agrobacterium* isolate SPI-2B-2 (GUS+), lanes 7 and 8 . *Agrobacterium* isolate SPI-2B-1 (GUS+), lane 9. 39-1 (NPTII+, GUS+, particle gun), lane 10. pGA482GG (NPTII+ control). Each lane was loaded with 10% (10 μl) of a 40-cycle reaction product.

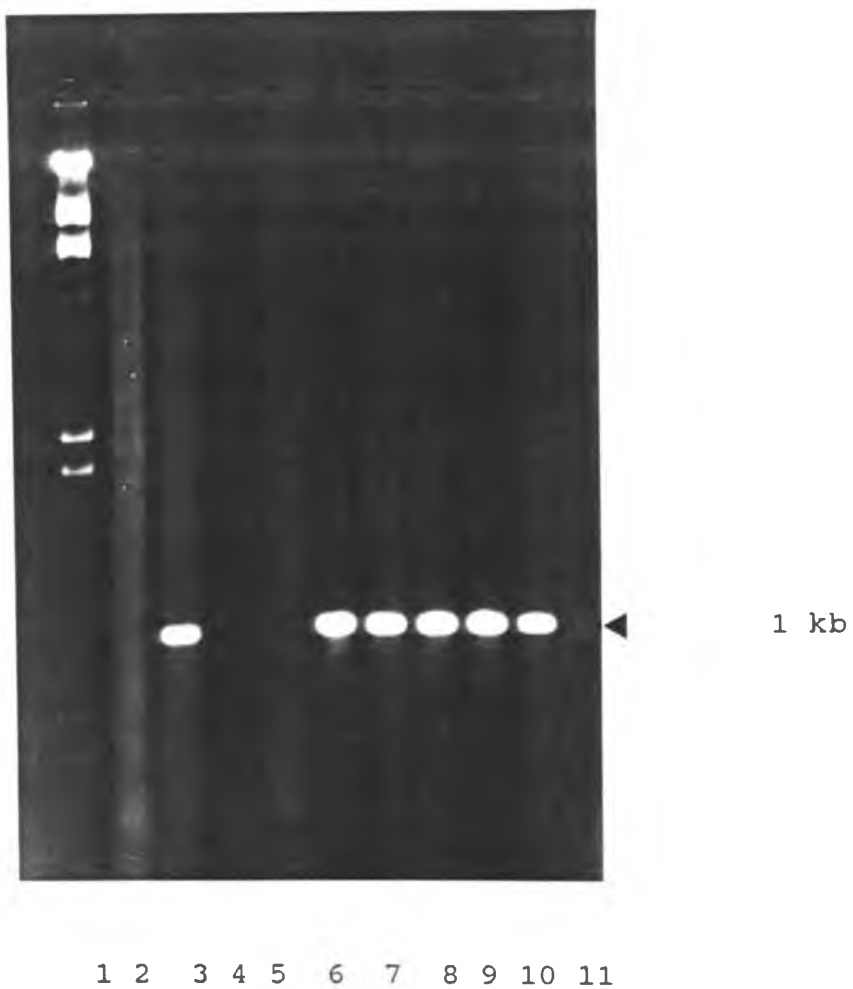

Fig. 7.3. PCR-amplified genomic DNA from untransformed and transgenic papaya leaves using PRV coat protein gene primers. Lane 1. *Hin*dIII lambda MW markers, lane 2. untransformed papaya, lanes 3 and 10. 55-1 (NPTII+, GUS+, particle gun), lanes 4 and 5. *Agrobacterium* isolate SAI-1D-1 (GUS+), lane 6. *Agrobacterium* isolate SPI-2B-2 (GUS+), lanes 7 and 8 . *Agrobacterium* isolate SPI-2B-1 (GUS+), lane 9. 39-1 (NPTII+, GUS+, particle gun), lane 11. 54-1 (NPTII+, GUS-, particle gun). Each lane was loaded with 10% (10 μl) of a 40-cycle reaction product.

isolates that had been transformed with CP-containing constructs (lanes 3 and 6 to 10).

In an experiment involving co-cultivation of embryogenic cultures of 'Kapoho' with *A. tumefaciens* strain A208.35, no selective growth was observed after more than a year of monthly subculturing on media containing 257 μM kanamycin. It appears that papaya was not a suitable host for this *A. tumefaciens* strain.

'Kapoho' embryogenic tissues were co-cultivated in a subsequent experiment with the C58 strain containing pGA482GG/cpPRV-4, pGA482GG/cpPRV-19-5, or the control plasmid, pGA482GG. The three-week recovery period was omitted, and selection pressure with 257 μM kanamycin was applied immediately after co-cultivation. Putative transgenic embryos became visible after four months; however, growth was very slow. In addition, some tissues removed from antibiotic medium after five months of culture with carbenicillin and cefotaxime became overgrown with bacteria. No selective growth was observed in the cultures initiated from suspensions. After nine months, cultures were transferred to MS medium containing only 209 μM kanamycin and growth of sectors was apparent.

High frequency somatic embryogenesis has been previously demonstrated in the culturing of immature zygotic embryos of papaya (Chapter 3) and hypocotyl sections (Chapter 4). Using these two regeneration systems

transgenic papayas were produced via microprojectile bombardment (Chapter 5). In the present study, embryogenic papaya cultures have been transformed with *A. tumefaciens* strain C58 and transgenic plants have been obtained. The A208.35 strain may also be suitable for papaya transformation, but not under the conditions attempted herein. With the *Agrobacterium* protocol, two methods are now available for tranforming papayas, both dependent upon embryogenic cultures for recovery of transgenic plants. Neither transformation method yielded transgenic plants when freshly explanted hypocotyl sections were the target tissues. In addition, suspension cultures of embryogenic calli were not suitable for selecting transgenic embryos following *Agrobacterium* co-cultivation.

The speed with which transgenic plants can be recovered differs between the two transformation systems. Transgenic embryos from *Agrobacterium*-mediated transformation appeared after six months compared to only four months after particle gun bombardment (Chapter 5). The difference may be partly attributed to overgrowth of cultures with *Agrobacterium* after co-cultivation. In addition, selection in the *A. tumefaciens* system may have required a lower concentration of kanamycin than was used with the particle gun-transformed cultures.

Differences between transgenic isolates produced by the two transformation systems may be interesting. Already

there are dissimilarities. The number of isolates recovered following the first co-cultivation experiment was low; however, so far, all isolates tested have expressed the GUS reporter gene, whereas only about one third of the kanamycin-resistant isolates produced via the particle gun expressed GUS and showed evidence for PRV coat protein gene integration into the papaya genome (Chapter 5). The apparent loss of *gus*A and the coat protein gene in the transformants produced by bombardment suggests rearrangement of genes (Klee et al. 1987, Klein et al. 1988b) or fragmentation of the plasmid. Three *Agrobacterium*-transformed isolates have shown evidence for integration of *kan* sequences, but in the isolate (SAI-1D-1) transformed by pGA482GG, the PCR amplification product is a little larger than the sequences amplified in plants transformed with *A. tumefaciens* containing pGA482GG/cpPRV-4 (SPI-2B-1 and SPI-2B-2). The sequences amplified in the latter examples appear to be identical in size to those amplified in all particle gun-transformed isolates observed. It will be interesting to compare the amplification products of *kan* and coat protein sequences from *Agrobacterium*-mediated and particle gun-mediated transformation to assess the efficiency of gene delivery between the two systems.

CHAPTER 8. SUMMARY

The three objectives of these experiments, 1) to develop an efficient regeneration system for papaya tissue culture, 2) to transform the regenerable papaya cultures and produce transgenic plants, and 3) to confirm and characterize the transgenic plants, have been achieved. Two efficient regeneration systems for papaya tissue cultures have been developed (Chapters 3 and 4), and transformed papaya plants that express chimeric foreign genes (Chapters 5, 6, and 7) have been regenerated. Some of the transgenic plant lines have been characterized, and the genes were shown to be stably inserted into papaya genomes (Chapters 6 and 7).

The regeneration systems were critical for recovery of transgenic plants. Regeneration procedures described in the literature for organogenesis (Arora and Singh 1978a, Litz et al. 1983) of papaya shoots were attempted. These methods were most closely analogous to the highly successful leaf disk method for *Agrobacterium*-mediated transformation (Gasser and Fraley 1989) developed by Horsch et al. (1985). The initial papaya regeneration experiments resulted in only three cultures bearing regenerants, and they all arose from somatic embryos (Chapter 4).

Two developments in transformation technology occurred during the progress of these experiments. First, the

demonstrations of *Agrobacterium tumefaciens*-mediated transformation of somatic embryos led to re-focussed effort in producing papaya somatic embryos rather than organogenic cultures. A critical experiment was the culturing of immature zygotic embryos on induction media containing 2,4-D (Chapter 3). Many of the somatic embryos developed directly from swollen apical dome and cotyledonary axil tissues, although others were produced indirectly from callus that developed on the radicle or from the shoot apex (Chapter 3). The importance of 2,4-D in the induction media, ranging in concentration from 2.3 to 113.1 μM, was corroborated in the culturing of seedling hypocotyl sections. Induction of embryogenic calli from hypocotyl explants, often in very high frequency (Chapter 4), provided a second source of somatic embryos.

The second important development in transformation technology that influenced this work was the advent of the particle gun. Somatic embryos that developed directly from the apical domes of zygotic embryos provided excellent targets for bombardment with the particle gun. Embryogenic callus produced from hypocotyl sections served as another target tissue, and transformation efficiency with these two embryogenic tissues was compared with a third type of tissue, freshly explanted hypocotyl sections. The low frequency of transformation achieved with bombardment of freshly explanted hypocotyls was in contrast to the

relatively high frequency of transformation with the bombarded zygotic embryos. Direct development of somatic embryos from meristematic apical domes probably accelerated isolation of transformants from bombarded zygotic embryos. Comparable transformation efficiencies were obtained in bombarded embryogenic calli. However, an important difference was found in the frequency of plant regeneration. The lower rate of plant recovery from embryogenic calli may be due to the longer period of culture on 2,4-D-containing media. The auxin-like compound 2,4-D is known to cause aberrations in chromosomes (D'Amato 1978) and in regenerated plants (Orton 1985). It is conceivable that papayas regenerated from the embryogenic callus cultures after a total of eight to ten months of exposure to 2,4-D may be adversely affected by the growth regulator. Zygotic embryos were exposed to 2,4-D for two months, at most. However, although control cultures of somatic embryos from 2,4-D-containing media regenerated slowly, they did not produce aberrant structures like some of the transgenic isolates. Therefore, gene transfer may have caused the disruption of normal morphology and 2,4-D may have impeded only the rate of regeneration.

Agrobacterium tumefaciens-mediated transformation of embryogenic calli and somatic embryos of papaya was more difficult than particle gun-mediated transformation because the bacterial re-growth problem required prolonged

treatment. The transgenic isolates did not appear as quickly as did those from particle bombardment, and they were recovered in smaller quantities. However, even with the small number of isolates recovered, it appeared that the *Agrobacterium*-mediated transformants expressed the full complement of genes that were delivered (Chapter 7). Compared with the particle gun-derived transgenic plants that showed GUS expression in only about one-third of the isolates, all of the *Agrobacterium*-derived isolates expressed GUS as somatic embryos and a greater proportion of them may provide plant lines possessing the desired gene constructs. The pale green isolates recovered from *Agrobacterium*-mediated transformation may have resulted from long-term culture of the target callus on 2,4-D-containing media prior to co-cultivation or the problem may stem from gene insertion. None of the particle gun-derived isolates lost the ability to produce chlorophyll; therefore, the response may be one exclusive to *Agrobacterium*-mediated transformation. Finally, the different fragment size obtained in PCR-amplification of *kan* DNA from one *Agrobacterium*-mediated transformant indicates that changes can occur in the tranforming DNA.

The development of transformation systems makes genetic engineering techniques available for papaya improvement. It is exciting that one of the GUS-expressing plant lines appears to be PRV resistant. Understanding genetic control

of the mechanisms plants use to survive adverse conditions such as pathogen attack and environmental stress may lead to further advances in crop improvement. Crop improvement, nurtured by classical disciplines of breeding and selection; application and management of water, nutrients and crop protective chemicals; management of soil, crop timing and population, has been targeted by practical applications of transformation. Transformation with genes that confer traits such as disease resistance may, in turn, provide new answers to questions in basic research. For example, some transgenic virus resistant plants that express the coat protein gene of one virus show broad spectrum resistance to other viruses. These results may be significant in elucidating the mechanisms that confer virus resistance or susceptibility.

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